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Genetic control of insect pest species – achievements, challenges, and perspectives

Edited by
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Genetic control of insect pest species – achievements, challenges, and perspectives

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Editorial: Genetic control of insect pest species—achievements, challenges, and perspectives

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Editorial on the Research Topic
Genetic control of insect pest species—achievements, challenges, and perspectives

Insect pests cause billions of dollars of losses in agriculture and livestock and hundreds of millions of disease cases every year due to the transmission of pathogens and parasites. Today, insecticide-based applications are still the most widespread strategy for controlling insect pests and disease vectors, and in many cases are the only effective solution available. At the same time, however, an increasing development of resistance to the main substance classes is being observed worldwide and in many different species. Moreover, the unacceptable impacts of insecticides on human health, non-target species, and the environment and biodiversity has become a major concern in recent decades. Alternative control approaches for insect pests that are effective, sustainable and species-specific are therefore in high demand.

Genetic control is a type of biological control and a promising approach to regulate insect pest populations in a species-specific manner. It is based on targeting the reproductive capacity of the target pest species to reduce population size to non-critical levels. The best known and also very successful genetic control strategy is the Sterile Insect Technique (SIT), which entails the continuous mass-release of irradiation-sterilized males of a given species to produce infertile matings in the field, leading to the decline in the target population over time. To date, SIT is only available for a few species, as its transfer to new target species is challenging and time consuming. Key aspects of this classical SIT and challenges in applying it to new pest species include mass rearing of target species, mass removal of female insects prior to irradiation and release, the sterilization procedure, and the biological quality control of the sterile insects produced.

Besides this classical SIT strategy, current research efforts are also focused on the development of genetic control approaches based on transgenic, symbiont-mediated, or gene-drive strategies. Modern genetic technologies offer new solutions for the improvement of existing genetic control strategies and insect strains, for faster and easier transfer of existing strategies to new target species, and also for the development of new genetic control

approaches. Publications within this Research Topic address pressing questions and challenges related to the genetic control of insect pests.

Several control approaches rely on the manipulation of genes of the sex determination pathway, for example, to create strains in which females carry a dominant lethal or strains in which the sex ratio is skewed towards males, both with the aim of suppressing a target population upon release of such insects. Such strategies require detailed knowledge of the sex determination pathway and the genes involved. In their review "Manipulation of Insect Sex Determination Pathways for Genetic Pest Management: Opportunities and Challenges," Sidall et al. highlights how knowledge of sex determination in target pest species is essential for all phases of development of control technologies. Belavilas-Trovas et al. address the reproductive capacity of mosquito females from a completely new angle by studying the involvement of long noncoding RNAs in *Aedes albopictus* reproduction, which could open a new avenue for insect pest management

The important area of sex separation ("sexing") for male-only releases was addressed within this Research Topic. Sexing may involve the removal of females during mass rearing by killing them or by sex sorting based on a male- or female-specific marker. Yamamato et al. produced several sexing strains in the agricultural pest *Drosophila suzukii* carrying a double female-specific lethal construct at different genomic positions to eliminate females before irradiation and evaluated their sexing performance as well as general fitness and male sexual competitiveness. One of their aims was to generate strains that can be tested against strains with different genetic backgrounds and they identified promising lines that can be used in such population suppression experiments.

Once such sexing strains are considered for application in the field, their functionality and performance in the local genomic background of the release area must be verified. Augustinos et al. evaluated the stability of *Aedes aegypti* sexing strains carrying a red marker and a recombination-suppressing inversion in six different genomic backgrounds. The same sexing traits were introgressed into the genomic background of the Northern areas of Pakistan's KP Province and tested for their genetic stability, biological quality, and their potential to be used for SIT applications against *Ae. aegypti* populations in Pakistan by Misbah-ul-Haq et al.

Another key aspect of genetic control strategies is the biological quality of the insects produced. The success of genetic control programs depends heavily on the dispersal rate, longevity, and mating success of released males. These parameters are influenced by conditions and procedures of mass rearing and irradiation, as well as by the transport of insects to release sites. Therefore, quality assessment and improvement of the insect strains and produced males are important aspects to ensure successful application. Several publications on this Research Topic address these important questions.

In order to reliably evaluate and, more importantly, compare the quality of insect strains and males produced, standardized and universally applied methods are needed. To test the flight ability of mosquito males, IAEA/FAO developed a flight test device that was further optimized and standardized in the study by Maiga et al. to ensure quality control of mosquito males. Yamada et al. used this flight test device to investigate how immobilization of mosquitoes during irradiation affects one of the standard parameters for male quality, flight ability. Mosquitoes are commonly immobilized during irradiation by chilling or anesthetics (nitrogen) to reduce damage caused by movement in confined spaces. They also tested longevity and evaluated the irradiation dose response in *Aedes* mosquitoes in combination with chilling or nitrogen exposure.

Quality effects in a transgenic sexing strain was also examined. One technology used to create sexing strains in several insect species is the tetracycline-off system. It consists of a lethal gene cassette whose expression can be controlled by the presence of the antibiotic tetracycline or doxycycline. Yan et al. studied the parental and transgenerational effects of these antibiotics in combination with the genomic position of the transgene in *D. suzukii*.

Insect performance must always be tested outside the laboratory setting and standard test conditions, i.e., in the field. Velo et al. conducted mark-release-recapture studies on irradiation-sterilized *Aedes albopictus* males produced in Italy and shipped to Albania to estimate, under field conditions, their dispersal capacity, probability of daily survival and competitiveness, and target population size in a highly urbanized area. Using sterile males from the same production facility, Balestrino et al. investigated how environmental factors and weather parameters affect the dispersal rate of released males in Italy. In another publication, Balestrino et al. investigated the effect of irradiation from a completely different angle: the effects of irradiation on vector competence. They compared the virus load and transmission efficiency in two *Aedes* species, *Ae. aegypti* and *Ae. albopictus*, with or without the irradiation dose of 40 Gy.

Gene drives offer another option to suppress insect pest populations, e.g., by driving a lethal trait into the natural population. Because this trait spreads with super-Mendelian inheritance, only small release numbers are required, largely eliminating the need for mass rearing when gene drive approaches are used. Moreover, gene drives can be used to replace natural populations, e.g., by introducing mosquitoes that are refractory to disease transmission, thereby maintaining the natural role of mosquitoes in food webs but abolishing vector capacity. In recent years, many different gene drive architectures have been developed, each bringing its own advantages and challenges. Gene drives can be powerful transformative technologies, and thus their potential application in the field also raises various concerns about how precisely such technologies can be controlled spatiotemporally, and what unintended consequences might result from their use. Verkuijl et al. address these Research Topic in their review entitled "Challenges in Developing Efficient and Robust Synthetic Homing Endonuclease Gene Drives," while Chennuri et al. describe the current state of the art genetic approaches for controlling CRISPR-based autonomous homing gene drives. Gene drive constructs tend to be large and complex and consist of multiple different elements, making it difficult to generate new gene drives and test the behavior of the different functional modules. Integral gene drives, characterized by a modular design, have been developed, allowing step-wise testing of the gene drive components before they become fully autonomous drives. In the current study Nash et al. took the next step and constructed an autonomous integral drive using intronic guide RNAs.

In addition to safety concerns regarding gene drive releases, there are also ethical questions surrounding the advancement of the technology. The development and release of gene drives involves

many different stakeholders, and one important Research Topic is the co-development of the technology with local stakeholders and communities and the reduction of asymmetry between developers and end-users. Kormos et al. discuss Research Topic with true stakeholder involvement and co-development, particularly with respect to stakeholders in low-income countries.

While new genetic tools and technologies promise solutions to many pressing problems in the sustainable and environmentally friendly control of insect pests, any release of genetically modified organisms into the environment requires a thorough evaluation and risk assessment of each genetically modified insect strain that is to be released. Alcalay et al. investigate the probability of an X-shredder relocation from an autosome to the Y chromosome. X-chromosome shredding is a mechanism to induce sex ratio distortion by biasing spermatogenesis towards Y-bearing gametes. Relocation of the X-shredder to the Y-chromosome could therefore result in invasive meiotic drive element.

Finally, despite rapid advances in genetic engineering in many different insect species and the rapid adaptation of new technologies such as CRISPR/Cas genome editing, not all insect orders are equally amenable to these technologies. One order that is lagging behind is the order Hemiptera. Pacheco et al. review the progress, challenges, and perspectives of gene editing and genetic control of hemipteran pests.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Potential for a Released Autosomal X-Shredder Becoming a Driving-Y Chromosome and Invasively Suppressing Wild Populations of Malaria Mosquitoes

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Sex-ratio distorters based on X-chromosome shredding are more efficient than sterile male releases for population suppression. X-shredding is a form of sex distortion that skews spermatogenesis of XY males towards the preferential transmission of Y-bearing gametes, resulting in a higher fraction of sons than daughters. Strains harboring X-shredders on autosomes were first developed in the malaria mosquito *Anopheles gambiae*, resulting in strong sex-ratio distortion. Since autosomal X-shredders are transmitted in a Mendelian fashion and can be selected against, their frequency in the population declines once releases are halted. However, unintended transfer of X-shredders to the Y-chromosome could produce an invasive meiotic drive element, that benefits from its biased transmission to the predominant male-biased offspring and its effective shielding from female negative selection. Indeed, linkage to the Y-chromosome of an active X-shredder instigated the development of the nuclease-based X-shredding system. Here, we analyze mechanisms whereby an autosomal X-shredder could become unintentionally Y-linked after release by evaluating the stability of an established X-shredder strain that is being considered for release, exploring its potential for remobilization in laboratory and wild-type genomes of *An. gambiae* and provide data regarding expression on the mosquito Y-chromosome. Our data suggest that an invasive X-shredder resulting from a post-release movement of such autosomal transgenes onto the Y-chromosome is unlikely.

Keywords: gene drive, malaria, sex-ratio distortion, genetic control, risk assessment

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INTRODUCTION

Mosquito species of the *Anopheles gambiae* complex are the main vectors of human malaria and pose an enormous burden on global health and economies (World malaria report, 2020). The progressive spread of insecticide resistant mosquitoes (Hyde, 2005; Sinha et al., 2014) has prompted the development of new methods to control these mosquitoes (Windbichler et al., 2008; Kyrou et al., 2018; Carballar-Lejarazú et al., 2020). One of the most promising is genetic control, which is based on the release of laboratory-modified insects into the environment. Released individuals mate with wild insects and transmit control traits that can suppress or modify the targeted population (Hamilton, 1967; Curtis, 1968). Among these, the most commonly used approach to genetically control insects has been the mass release of sterile males—the so-called Sterile Insect Technique (SIT) (Knipling, 1959; Wyss, 2000). When wild monandrous females mate with released sterile males, their eggs are fertilized by sperm carrying mutations that abort embryo development. If sufficient numbers of sterile males are released over a long enough period, the wild population can be effectively suppressed or even eradicated. However, the economic costs of an SIT program that aims for mosquito suppression in very large areas and the need to maintain sterile releases indefinitely, have restricted the implementation of this method to date [but see; (Zheng et al., 2019, Hendrichs et al., 2021; Balatsos et al., 2021)].

One way to improve the efficiency of such approaches is through the release of fertile males that are daughterless. Since male mosquitoes do not contribute to disease transmission, releasing males that have viable and fertile sons can help to temporarily maintain the frequency of the allele or transgene in the population, which in turn helps to reduce the abundance of females. Two strategies based on such fertile males have been developed in mosquitoes thus far: fs-RIDL (for female-specific Release of Insects carrying Dominant Lethals) and sex ratio distorters based on X-chromosome shredding (Thomas et al., 2000; Burt, 2003; Phuc et al., 2007; Windbichler et al., 2007; Galizi et al., 2014). fs-RIDL is based on a construct that is lethal to females that inherit it, so that daughters of released transgenic males born in the field and inheriting the transgene die before maturing or are unable to fly (flightless), but sons survive and pass the transgene to their offspring (Thomas et al., 2000; Phuc et al., 2007). Sex-ratio distortion based on X-chromosome shredding instead, relies on the expression of a sequence-specific endonuclease during male spermatogenesis that recognizes and cleaves sequences that are both specific and abundant on the X-chromosome (Windbichler et al., 2007; Galizi et al., 2014). As a result, X-chromosome-bearing gametes are eliminated from the viable sperm population, thus biasing offspring sex-ratios towards males (Burt, 2003; Deredec et al., 2008; Papathanos and Windbichler, 2018; Haghighat-Khah et al., 2020). Mathematical models predict that both approaches are more efficient than SIT in terms of the number of modified males that need to be released to achieve a similar level of population suppression (Schliekelman et al., 2005; Burt and Deredec, 2018). Despite being more efficient, both fs-RIDL and autosomal X-shredders (where the transgene is located on an autosome) are self-limiting. The transgenic constructs underlying the phenotype will therefore not spread in the population, because they are inherited in a Mendelian fashion and do not provide any fitness advantage over the wild type. This is different for self-sustaining approaches such as those incorporating gene drive constructs (Alphey, 2014; Hammond and Galizi, 2017). The fact that in X-shredding, the X-chromosome-bearing gametes are eliminated pre-zygotically can be used for self-sustaining genetic control applications, in the form of Y-chromosome drive as originally proposed by Hamilton (Hamilton, 1967). This could be done by linking a functional X-shredder to the Y-chromosome, in which case both the Y-chromosome and the X-shredder gain a transmission advantage through preferential inheritance of male-forming gametes (Deredec et al., 2011).

A X-shredding sex-distorter was first developed in *An. gambiae* by Galizi et al. (2014). They used variants of the I-*Ppo*I endonuclease that cut a specific DNA target sequence within the 28S ribosomal DNA locus, which in *An. gambiae* is located exclusively on the X chromosome in approximately 200–400 copies (Collins et al., 1989). These I-*Ppo*I variants were fused to eGFP and driven by the *An. gambiae beta-2 tubulin* regulatory regions, which become active in primary spermatocytes entering male meiosis (Catteruccia et al., 2005). The resulting transformation constructs also included the *DsRed* transformation marker driven by the neuron-specific *3xP3* promoter, and the entire cassette was flanked by piggyBac-specific left and right arms containing the inverted terminal repeat sequences (ITRs) (**Figure 1A**). Of all the transgenic strains examined, ^{gfp}124L-2, since renamed by the Target Malaria Research consortium as Ag(PMB)1 (for *An. gambiae* Paternal Male Bias strain 1) expressing the I-*Ppo*I structural variant W124L, showed high sex ratio distortion among progeny of transgenic males (approximately 95% males), without significantly impairing male fertility and fitness and is thus being currently evaluated for field testing by the Consortium (Galizi et al., 2014). Inverse PCRs produced as part of that study showed an autosomal location of the transgene, from where the sex-distortion phenotype was stably inherited over consecutive generations. In large cage experiments, weekly inoculative releases of transgenic Ag(PMB)1 males led to a reduction both in the egg productivity of the population and the frequency of females over successive generations consistent with model predictions (Facchinelli et al., 2019).

Since Ag(PMB)1 males are fertile, their release should result in viable offspring in the field, unlike sterile males. This would provide invaluable information about how transgenic, laboratory-reared males of *An. gambiae* disperse spatially once released and can be achieved even from small-scale releases aimed at capacity-building and methodology development. Unlike fs-RIDL strains that were directly developed for deployment, the lack of a conditional expression system or some other design to control activity of the X-shredder makes this strain unsuitable for large-scale programs aiming directly for population suppression, since rearing at large numbers is logistically difficult because this strain can only be maintained through females to avoid strain loss. Moreover, the Ag(PMB)1 X-shredder was not designed for

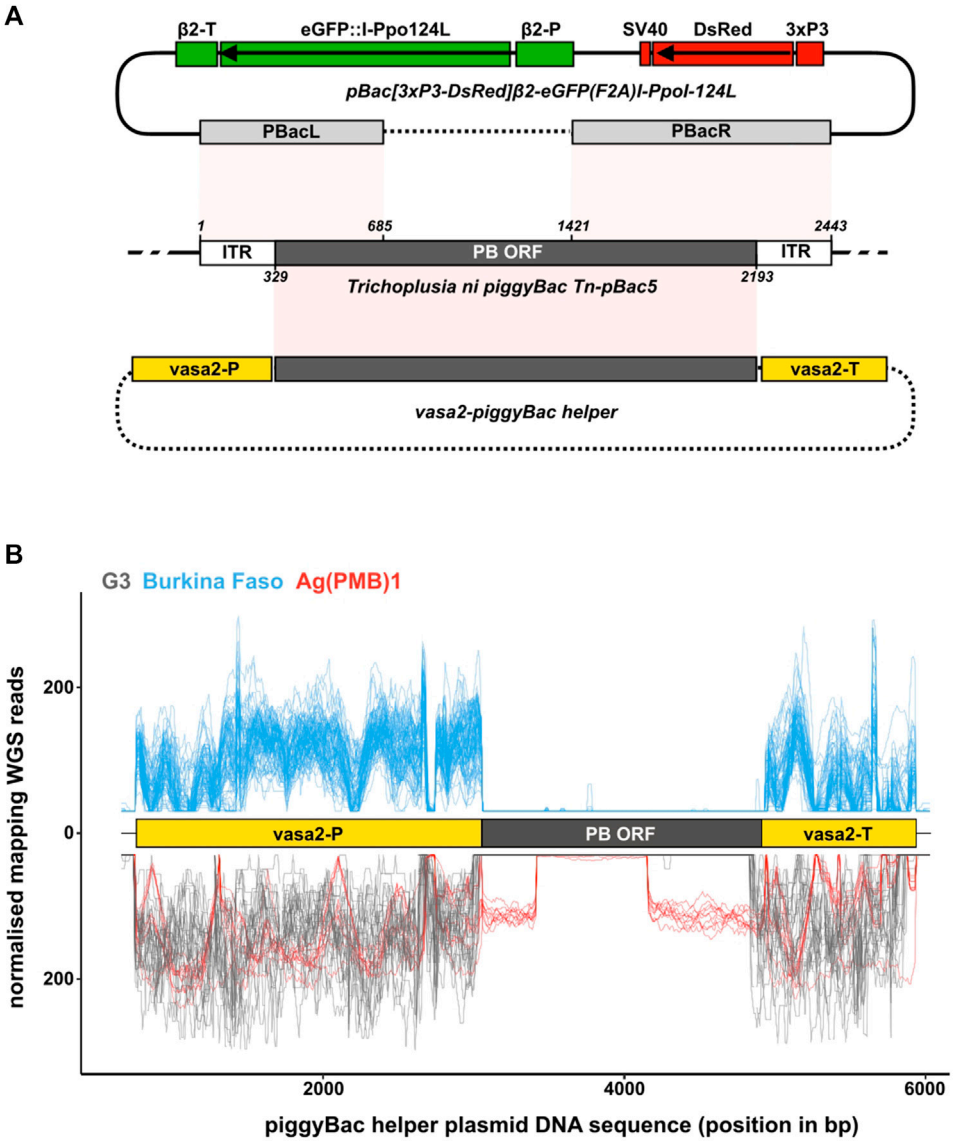

FIGURE 1 | piggyBac transposase components in laboratory and wild type individuals. **(A)** Schematic of a wild type piggyBac (PB) transposon from *Trichoplusia ni* (middle; NCBI accession DQ236240.1), the Ag(PMB)1 transformation construct [(Galizi et al., 2014); top] and the PB helper plasmid [(Volohonsky et al., 2015); bottom]. Shown are the regions of the endogenous PB locus present in the two microinjected plasmids highlighting how both the transformation and helper plasmid lack the complete machinery required for transposon mobility. pBacL **(left)** and pBacR **(right)** arms present in the pBac[3xP3-DsRed]β2-eGFP:I-*Ppo*I-124L transformation construct contain the entire flanking inverted terminal repeats (ITRs) and partial regions of the PB open reading frame (ORF). The helper PB plasmid containing the complete PB ORF driven from the *vasa2* regulatory regions lacks the flanking ITRs. Sequences of the transformation construct that are integrated in the genome, and those present only transiently in injected individuals are shown in solid lines and dashed lines, respectively. **(B)** Mapping of whole genome sequencing reads from the G3 and Ag(PMB)1 controls (**bottom** in negative y-axis; grey and red) and the 81 wild type individuals collected in Burkina Faso villages (**top**; blue). The position of the *An. gambiae vasa* regulatory regions (yellow boxes) and the PB transposase ORF (black box) is also shown. Reads are normalized by scaling counts to the number of reads in the most abundant sample.

gene drive in its current form - for example it is not able to home into targeted sequences. It also does not display any fitness advantage over wild type mosquitoes. Consistent with this, models predict that the Ag(PMB)1 transgene would disappear over time when releases are discontinued (Burt and Deredec, 2018) and recent large cage experiments confirmed the loss of the transgene from the population over time (Pollegioni et al., 2020).

One feature that is unique to an autosomal X-shredder compared to other self-limiting strategies, is the possibility that it could move to the Y-chromosome after release, potentially becoming self-sustaining in the form of a Y-chromosome drive as first coined by Hamilton (1967). If active, a Y-chromosome linked X-shredder could directly benefit from the increased transmission of the Y-chromosome

due to preferential inheritance of male-forming gametes, thus increasing in frequency, persisting longer and dispersing further than initially planned. The sequential events required for such a driving Y to occur can be mapped to "pathways to harm" using a problem formulation approach adopted widely in environmental risk assessments (**Supplementary Figures S1, S2**). Three requirements must be fulfilled for a driving Y to occur: 1) the autosomal X-shredder must first move from its original autosomal position and become physically linked to the Y-chromosome; 2) the X-shredder would need to be expressed from its new position on the Y-chromosome during late spermatogenesis in a spatiotemporal manner that is similar to its original expression from the autosome; and 3) it should impart no significant cost to male fertility or male viability as a result of its new Y-chromosome-linkage (**Supplementary Figures S1, S2**).

With regard to requirement 1), excluding any DNA repair mechanisms as this would require unlikely pairing of the autosome and the Y chromosome, there are two possible mechanisms that could result in an autosomal transgene moving to the Y-chromosome: 1) a transposase-mediated transposition to the Y-chromosome of the piggyBac (PB) transposable element that was used to create the transgenic strain, or 2) a recombination-mediated reciprocal translocation resulting in large chromosomal rearrangements between the autosome and the Y-chromosome. Of the two mechanisms, translocation is the less likely route, because translocations between autosomal segments and the Y-chromosome occur very rarely in nature (see Discussion). On the other hand, transposition from the autosome to the Y through the remobilization of the PB transposon could be possible, if the X-shredder transgene co-occurs in a genome containing an active PB transposase.

In this paper, we addressed the possibility of transgene remobilization by examining mosquito genomes for evidence of the PB transposase which recognize the inverted terminal repeats of the transgene and remobilize it. With regard to requirement 2), expression from the Y–we generated two independent transgenic strains containing the eGFP:I-*Ppo*I-124L X-shredder construct on the Y-chromosome and evaluated the level of expression and sex-ratio distortion. Finally, we discuss implications for male fertility depending on the route of movement to the Y-chromosome.

RESULTS

Evaluating the Remobilization Potential of the Autosomal Ag(PMB)1 X-Shredder Transgene

The Ag(PMB)1 strain was generated by Galizi et al. (2014) by micro-injecting *An. gambiae* G3 embryos with a mixture of the transformation plasmid [pBac(3xP3-DsRed)β2-eGFP:I-*Ppo*I-124L] and a helper plasmid, containing the piggyBac (PB) transposase expressed from the *vasa* regulatory regions (Volohonsky et al., 2015), which direct expression in germline tissues (**Figure 1A**) (Papathanos et al., 2009). By providing the PB transposase in *trans* from a transiently co-injected helper plasmid, the transformation construct itself became immobilized once it integrated in the genome. This is because, unlike the complete PB transposable element, the transgene lacks the complete transposase enzyme that is required for remobilization. Therefore, integrated PB transgenic constructs can only be remobilized in mosquito transgenic strains, if a PB transposase source is available (O'Brochta et al., 2011).

To assess the stability of the Ag(PMB)1 transgene, we first evaluated whether the original insertion site, as described in Galizi et al. (2014), has remained stable in the approximately 100 generations since its initial generation in laboratory populations that are typically maintained by crossing transgenic females (approximately 200 per generation) to wild type males. We designed PCR primers that span the PB transgene and genomic boundary (flanking regions) as originally reported (Galizi et al., 2014), and repeated the PCR using genomic DNA from 162 heterozygous transgenic individuals, that were generated by crossing transgenic females to wild type males. Transgene inheritance on the basis of DsRed fluorescence was scored twice during larval development and found in half of larval offspring, as expected for a single copy of the transgene in the genome. Of the 162 transgenic individuals tested, all contained the transgene in the expected location, as indicated by successful amplification from primers annealing in internal and flanking sequences (**Supplementary Figures S3, S4**). These results suggest that either the Ag(PMB)1 transgene has not remobilized in the strain, or, if new alleles have emerged, that these are not represented at detectable levels using our laboratory assays designed to test the transgene location. This indicates that PB transposase does not occur naturally in the genome of the laboratory colony. It also suggests that none of the other naturally occurring transposable elements present in this strain are able to remobilize the Ag(PMB)1 transgene, in the absence of the initially provided PB-helper source.

Given this issue of detection at scale, we next tested whether we could detect the gene encoding PB transposase in the genomes of the G3 or Ag(PMB)1 strains. This would exclude the possibility that PB transposase gene is present but is either non-functional, e.g., through mutations in its open reading frame, or suppressed by gene silencing by piRNAs (Senti and Brennecke, 2010; Halic and Moazed, 2009). To do this, we generated whole genome sequence (WGS) libraries from genomic DNA extracted from 10 individuals (five females, five males) of the Ag(PMB)1 strain and downloaded WGS libraries from 24 previously sequenced G3 individuals from the same insectary colony (PRJNA397539). We mapped the WGS data to the PB helper plasmid that was originally used to generate the Ag(PMB)1 transgenic strain, containing the PB transposase driven by the 5′ and 3′ regulatory regions of *An. gambiae vasa* gene. Mapping WGS reads against the helper plasmid ensured that the coding sequence evaluated is experimentally verified to catalyze excision of PB transgenes, instead of a different transposable element that may be related at the sequence level but is unable to excise PB transgenes. The helper plasmid included internal positive controls, in the form of regulatory sequences from the endogenous single-copy *vasa* gene and parts of the flanking

PB left and right arms of the Ag(PMB)1 transgene (**Figure 1A**). We observed a high number of mapping WGS reads from G3 samples against the *vasa*-derived regulatory sequences on the helper plasmid, but no continuous mapping in the region corresponding to the PB transposase enzyme (**Figure 1B**). For the Ag(PMB)1 strain, genomic reads mapped to both the endogenous *vasa* regulatory sequences and to internal sequences of the PB ORF that correspond with the parts of PB left and right arms included in the transformation construct used to generate Ag(PMB)1, as expected (**Figure 1A**). No reads were detected on the PB coding sequence that is excluded in the transformation construct (**Figures 1A,B**). We then repeated the same analysis using single-mosquito WGS data from 81 field-caught individuals collected in Burkina Faso in 2012 (NCBI BioProject Accession PRJEB1670), which is considered for a potential release of Ag(PMB)1 mosquitoes by the Target Malaria Consortium (**Supplementary Figure S5**) (Scudellari, 2019). Similar to the results from the G3 samples, no reads mapped to the part of the helper plasmid encoding the PB transposase open reading frame with reads mapping exclusively to the regions of the endogenous *vasa* gene. Together, these results suggest that the PB transposase is unlikely to be in the local genetic background of populations into which an introgressed autosomal Ag(PMB)1 transgene may be released in the future.

X-Shredder Expression From the Y-Chromosome During Spermatogenesis

The second requirement for the Ag(PMB)1 X-shredder to display gene drive and invasiveness, assuming the transgene has first moved to the Y-chromosome, is that it is expressed in a correct spatiotemporal manner and level from its new location. In the Ag(PMB)1 strain, X-shredding is achieved through the expression of the eGFP:I-*Ppo*I-124L transgene from the *An. gambiae beta2-tubulin* regulatory regions, which is highly active shortly before the first meiotic division in primary spermatocytes, and continues throughout the subsequent stages of spermatozoa differentiation (Michiels et al., 1993). In previous work, we have shown that transgenes driven from this promoter are strongly expressed when located on *An. gambiae* autosomes, but when they are inserted on the X-chromosome, expression is undetectable (Magnusson et al., 2012). This includes various X-chromosome-linked X-shredder variants, where no significant expression or sex bias was observed (Galizi et al., 2014). Similar observations of X-linked transgene transcriptional suppression around meiosis have been made in other species (Hoyle et al., 1995; Hense et al., 2007; Kemkemer et al., 2014). This phenomenon, called meiotic sex chromosome inactivation (MSCI), is thought to be one of the main driving forces leading to the observed paucity of sperm-specific genes on the X-chromosome, both in *An. gambiae* mosquitoes and in other species (Magnusson et al., 2011; Papa et al., 2017; Taxiarchi et al., 2019). By comparison, much less is known about transgene expression during spermatogenesis from the *An. gambiae* Y chromosome, which is estimated to be around 26 Mbp long, approximately 10% of the mosquito genome (Bernardini et al., 2017), and is composed nearly entirely of a few massively amplified, tandemly arrayed repeats and five known genes (Hall et al., 2016).

To test whether MSCI has a similarly inhibitory effect on transgene expression during spermatogenesis on the Y-chromosome as the X-chromosome, we generated two independent transgenic strains harboring the Ag(PMB) 1 X-shredder, eGFP:I-*Ppo*I-124L, on the *An. gambiae* Y-chromosome. The first transgenic strain, called YpBac-β2-^{gfp}124L, was generated by random PB integration. We sequenced the insertion site of the YpBac-β2-^{gfp}124L transgene by inverse PCR on genomic data extracted from transgenic males and found that the construct had inserted within the highly-abundant Y-chromosome-specific transposable element *zanzibar* (Hall et al., 2016) (**Figure 2A**). The second transgenic strain, called YattP-β2-^{gfp}124L, was obtained by secondary φC31 site-specific integration into an AttP docking site we previously inserted on the Y-chromosome (Bernardini et al., 2014). Similar to the YpBac-β2-^{gfp}124L strain, the AttP site is located in a region of the Y-chromosome containing the *zanzibar* repeat, though it is not possible to estimate the distance between these two insertions given the lack of a continuous Y-chromosome genome assembly (Hall et al., 2016).

As would be expected from Y-linked insertions, transgenic offspring from males of both Y-linked strains and wild-type G3 females were exclusively males. Testes from transgenic males of both strains displayed no obviously detectable eGFP signal by fluorescence microscopy above background auto-fluorescence, which would be expected if the eGFP:I-*Ppo*I-124L X-shredder transgene was expressed (**Figure 2B**), and overall testes fluorescence was indistinguishable from testes of wild type males in all individuals tested over multiple generations. These strains have, and still are under observation for Y-expression. In total, hundreds of observations have been made over more than 40 generations since they were generated. Conversely, expression from the 3xP3-DsRed transformation markers in both strains was phenotypically indistinguishable from autosomal insertions, suggesting that Y-chromosome linkage does not interfere with somatic expression of transgenes, at least from these two positions (Bernardini et al., 2014). To quantify this observation, we next analyzed the levels of eGFP:I-*Ppo*I-124L transcription in the testes of the YattP-β2-^{gfp}124L and YpBac-β2-^{gfp}124L strains. As a control, we also evaluated expression in testes of two additional strains from the Galizi et al. (2014) study [^{gfp}124L-3 and ^{gfp}111A-2, called here Ag(PMB)2 and Ag(PMB)3, respectively] and wild-type males. Results from the quantitative RT–PCRs show no significant levels of eGFP:I-*Ppo*I-124-L expression in the testes from both Y-linked transgenic lines compared to wild type testes and to eGFP:I-*Ppo*I-124-L expression in the Ag(PMB)1-3 strains (**Figure 2C** and **Supplementary Table S1**). Consistent with the lack of X-shredder expression, we did not detect any significant sex bias, compared to the expected 50%, among progeny when transgenic males from each Y-linked strain were crossed to wild type G3 females (Y-pBac124L; $\chi^2 = 12$, $p > 0.05$, Y-AttP124L; $\chi^2 = 12$, $p > 0.05$; **Figure 2A**; **Supplementary Table S2**). These results highlight that, as would be expected

A

Strain	Construct	Eggs per female	hatching %	% males	Insertion site
YpBac-β2-^{gfp}124L	pBac[3xP3-DsRed]β2-eGFP::I-PpoI-124L	N/A	N/A	54.6 ±2.9 n=13 (1179)	TGCTGAATTCTAAAA GTTTG**TTAA**ACGTAT ATTTGTATCTAGTT
YattP- β2-^{gfp}124L	attB[eCFP]β2-eGFP::I-PpoI-124L	135.5 ±8.5 n=19 (2574)	81.2 ±3.4 n=19	53.4 ±1.2 n=19 (1850)	TCATTGCTAACATGA AGGCA**TTAA**AAACAA AGGATACCAAGTCA

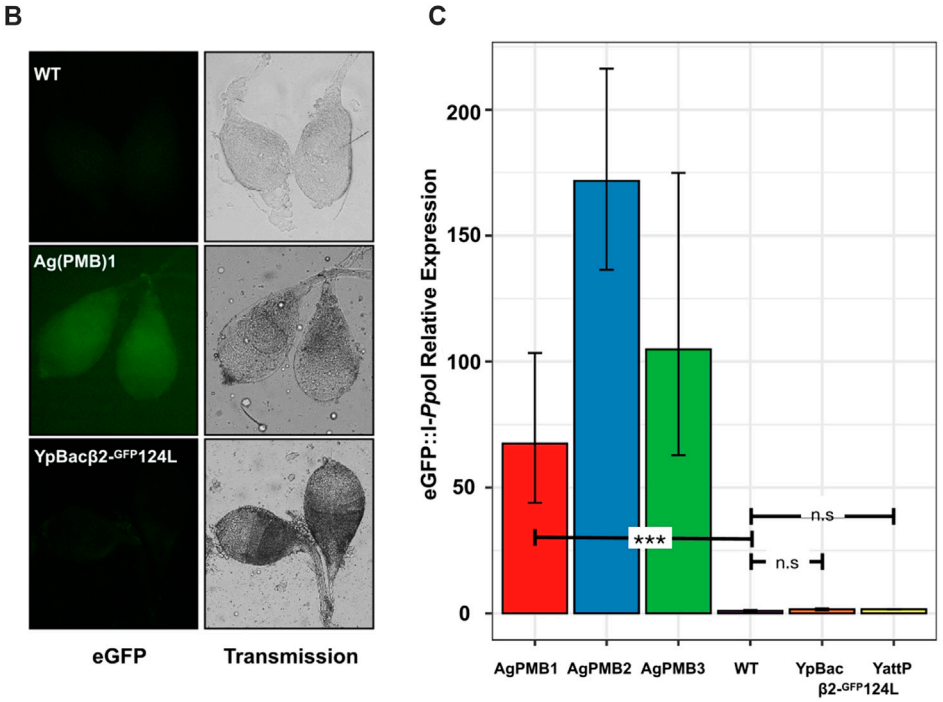

FIGURE 2 | Transcriptional suppression of Y-linked X-shredder constructs abolishes sex ratio distortion. **(A)** Progeny analysis of males from the two Y linked X-shredder strains crossed to wild-type females. Shown is the average number of eggs laid per n females analyzed (±represents the standard error of the mean; SEM). Average percentage of larvae hatching from the eggs (±SEM), from *n* females analyzed. Average percentage of males in the progeny (±SEM) from *n* females. The total number of eggs or individuals counted in each experiment is given in parentheses. Sequences (20 bp each side) flanking the PB integration site (TTAA) of the transformation constructs are also shown. **(B)** eGFP fluorescence from dissected wild type (WT), Ag(PMB)1 and YpBac-β2-^{gfp}124L testis. **(C)** Quantitative RT-PCR showing the relative expression of eGFP:I-PpoI variants in autosomal X-shredder strains [Ag(PMB)1-3] and Y-linked X-shredder strains. Expression levels were normalized to G3 wild-type (RQ = 1) which contains no I-*Ppo*I component. Expression of the X-shredder is undetectable in both Y-chromosome insertions compared to G3 wild-type (unpaired t-test $p = 0.1669$ for YpBac and $p = 0.2509$ for YattP). Expression levels from autosomal strains, Ag(PMB)1 (unpaired t-test $p = 0.0078$), Ag(PMB) 2 (originally W124L-3) and Ag(PMB)3 (originally L111A-2) which led to sex ratio distortion are shown.

from MSCI, the Y-chromosome is not permissive to transgene expression from the *beta2-tubulin* promoter during late spermatogenesis, similarly to the X-chromosome.

DISCUSSION

Genetic control strategies that aim to suppress wild populations of mosquito disease vectors have garnered significant interest, and field trials of a number of these systems, including classical SIT, IIT (*Wolbachia*-based sterility), and RIDL are now underway (Harris et al., 2012; Zheng et al., 2019). Synthetic sex-ratio distorters based on X-chromosome shredding have now been developed in *An. gambiae* (Galizi et al., 2014; Galizi et al., 2016) and more recently in *Drosophila melanogaster* and *Ceratitis capitata* (Fasulo et al., 2020; Meccariello et al., 2021). This system has been shown both theoretically and experimentally to be more efficient than classical SIT, in terms of the number of insects that need to be released (Schliekelman et al., 2005; Galizi et al., 2014; Burt and Deredec, 2018). In their most basic form,

autosomal X-shredder constructs are self-limiting, and their release can potentially result in local and limited suppression if sufficient males are released over a long enough period. Nonetheless, field releases of fertile autosomal X-shredder males have not yet been conducted.

Here, we have evaluated the theoretical possibility whereby an autosomal X-shredder could convert into a self-sustaining, driving Y-chromosome after release. The requirements for such an event to occur include: 1) movement of the X-shredder to the Y-chromosome; 2) its subsequent expression from the Y during late stages of spermatogenesis at a level that result in X-chromosome shredding; and, 3) have a low enough fitness cost to male carriers such that the X-shredding effect is net beneficial to males carrying such a Y-chromosome. We reason that there are two possible mechanisms that could result in the linkage of the autosomal Ag(PMB)1 to the Y-chromosome: 1) a transposase-mediated remobilization of the transgene and 2) a large chromosomal rearrangement resulting in the reciprocal translocation between the region of the autosome containing the transgene and the Y-chromosome.

We have evaluated the potential of remobilization of the Ag(PMB)1 transgenic construct through transposition, mediated by the intact PB inverted terminal repeats (ITRs) on either side of the transgene cassettes, which were used for the initial generation of the strain. Their presence makes it at least theoretically possible that the Ag(PMB)1 transgene could remobilize from its autosomal position, if a source of the PB transposase occurs in *trans*. We therefore evaluated whether the PB transposase is present in the genomes of the laboratory Ag(PMB)1 and G3 colonies, and also in field-derived samples from Burkina Faso. We found no evidence of the complete PB transposase coding sequence in any of the samples we sequenced, suggesting that PB is not present at appreciable frequencies in *An. gambiae* mosquitoes sampled from nature. This result is supported by the long-term stability of the Ag(PMB)1 insertion site over 100 generations since its original construction, a result that suggests that other naturally occurring repetitive elements in the genome of the Ag(PMB)1 strain, including those that appear as PB-like by genome-wide translated nucleotide searches, are not functionally capable of PB transgene remobilization. Long-term stability of transgenic insertions in *An. gambiae* laboratory strains in the absence of experimentally provided PB transposase is well known, including through directed efforts of enhancer trapping through transgene mobilization (O'Brochta et al., 2011). The computational methods we developed to screen for the presence of PB transposase in genome sequencing data from wild type, transgenic and field samples could be adapted in the future for high-throughput screening of sequenced samples collected from the field to identify and quantify the presence of transgenic alleles without the need for fluorescence microscopy or complicated molecular genotyping protocols.

We were not able to test the second possibility of remobilization by chromosomal translocation, as these occur very rarely during meiosis. In over 7 years of both standard laboratory rearing (Galizi et al., 2014) and large-scale multi-generational cage studies of the Ag(PMB)1 strain (Facchinelli et al., 2019; Pollegioni et al., 2020), translocations involving the autosomal transgene and the Y-chromosome have never been detected–an event that would be noticeable in defined crosses of individuals since fluorescent transgenic individuals would only be male. This extends to scaled experimental conditions, in which large numbers of Ag(PMB)1 individuals were screened by high-throughput sorting of individuals using the COPAS sorter based on the 3xP3-DsRed marker and then subsequently separated by sex at the pupal stage (Burt and Deredec, 2018). This is, in part, expected because of how rarely such events occur, meaning that experimentally verified rates for such events are not readily available for the Ag(PMB)1 strain. When translocations between autosomes and the Y are desired, they can be artificially induced in the laboratory, for example with ionizing radiation, chemical agents, or UV radiation. This is commonly done for insects to link selectable phenotypes to the Y-chromosome, in so-called genetic sexing strains (GSSs). GSSs are developed so that males can be separated from females on a large scale in insect bio-factories that produce animals for genetic control programs, such as SIT (Gilles et al., 2014). This is done by linking selectable traits, for example insect color or high temperature tolerance, to the Y-chromosome using induced reciprocal translocations of mutant alleles located on autosomes. Once generated, these GSSs are then maintained in large numbers, with billions of insects being produced weekly. The large colony size makes it possible to detect rare events that result in the breakdown of linkage between maleness and selectable trait. One of two ways this can happen is through a "reverse" reciprocal translocation involving the previously modified Y-chromosome and an autosome (known as a type 2 recombination event) (Franz et al., 2005). Because such events lead to a breakdown of the genetic sexing system and restore male fertility of the semi-sterile males (arising from the translocation itself) and leading to their accumulation, their occurrence is tightly monitored in large-scale rearing operations. In the only report that quantified the rate of type-2 recombination and distinguished it from type-1 (which does not involve the translocated Y- chromosome and is more common among the two) in a GSS of the Mediterranean fruitfly *Ceratitis capitata*, the rate was estimated to be 10^{-5} or less, i.e., occurring in less than 1 out of 100,000 male individuals (Franz et al., 2005). In this case however, there are two significant factors that would indicate that the rate of an initial, uninduced autosome-Y translocation would be much lower. First, the rate of recombination in the GSS describes an event reversing a previously induced autosome-Y translocation, which is likely to be largely mediated by homologous sequences that are now present on the two translocated Y fragments; a homology that does not normally exist between autosomes and the Y chromosome. Therefore, the expected recombination rate resulting in a reciprocal translocation between an autosome and Y would be lower. Second, the reversion of the previously translocated autosomal fragment on the Y restores male fertility, that was first compromised by the translocation to the Y (because of gamete chromosomal imbalance–discussed below) (Franz et al., 2005). This means that type-2 recombinant males will have more viable offspring increasing their rate of occurrence in

the population. Together, these factors suggests that the probability of a translocation event involving the autosomal Ag(PMB)1 transgene and the Y-chromosome in progeny of released males born in the field is expected to be much lower than 10^{-5}. This rate would depend on the size of the Y-chromosome and the relative rate of recombination in the male germline, which in *An. gambiae* is approximately 1.6 cM Mb^{-1} for autosomes (Pombi et al., 2006) and similar between males and females (Benedict et al., 2003).

In the unlikely event that the transgene was to move to the Y-chromosome, we provide data regarding the expression of the X-shredder from this chromosome, and conclude that MSCI during spermatogenesis does affect the Y-chromosome of *An. gambiae*. Our results from two transgenic strains harboring the Ag(PMB)1 X-shredder transgene in two different positions on the Y-chromosome, reveal transcriptional suppression during late spermatogenesis from the *beta2-tubulin* promoter, complementing our previous work which confirmed this for the *An. gambiae* X-chromosome (Magnusson et al., 2011; Galizi et al., 2014; Papa et al., 2017). We found no evidence of X-shredder expression by quantitative RT-PCR, nor by fluorescence microscopy of transgenic testis. Offspring of transgenic males from both Y-linked strains therefore had sex-ratios similar to wild-type males. Hence, even if the Ag(PMB)1 transgene successfully moved to the Y-chromosome by transposition (first requirement for a driving Y) it is unlikely that the X-shedder would be active. Since MSCI-factors regulating transcriptional suppression physically spread across the sex chromosomes after becoming localized on their unsynapsed axes (Ichijima et al., 2011; Ichijima et al., 2012), it is also expected that translocated autosomal fragments would become suppressed by MSCI during meiotic stages of spermatogenesis. Therefore, the weight of evidence argues strongly against the likelihood of movement of the Ag(PMB)1 transgene to the Y chromosome, particularly via transposition. However, the equally necessary prerequisite for a pathway to a driving Y, namely expression of the X-shredder on the Y chromosome during male meiosis, seems highly implausible based on the evidence presented here.

The final requirement for a Y-linked X-shredder to spread through populations is that its movement to the Y-chromosome and subsequent expression from it would have no significant fitness costs to males harboring it. Such fitness costs would counteract the theoretical advantage gained by the Y-linked X-shredder from increased transmission through elimination of X-bearing sperm. Among the factors determining these fitness costs, the largest contributors would likely be the mechanism leading to Y-linkage and the outcomes of this movement on each chromosome. Reciprocal translocations between an autosome and the Y-chromosome have be found to result in significant male fertility costs (Roukos and Misteli, 2014). Because of the simultaneous segregation of non-homologous centromeres (adjacent-1 segregation) during meiosis, only 50% of the offspring produced by males are genetically balanced, i.e., males are 50% sterile (Yamada et al., 2012). In certain cases, this semi-sterility can be even higher, for example in an *An. arabiensis* GSS showing 73.3% male sterility (Yamada et al., 2012). Therefore, a Y-linked X-shredder that arose through a translocation event would likely display sufficiently high male fertility costs that it would rapidly disappear from the population. For transposition-mediated Y-linkage male fitness costs cannot be predicted *a priori*, as gamete balance and genic content would depend on both the excision event (i.e., how much of the surrounding chromosome is excised) and on the integration position on the Y-chromosome (i.e., subsequent knock-out of genes essential for male fitness such as the male-determining gene).

In summary, the findings of the current study support the low probability of transgene remobilization from the autosome to the Y-chromosome. Moreover, even if such a rare event occurred, where the X-shredder would become linked to Y-chromosome, activity of the X-shredder at the required stage of spermatogenesis would likely be impeded via chromosome wide suppression of gene expression on meiotic sex chromosomes. Our results also show that prospects for the successful building of self-sustaining Y-linked X-shredders for mosquito control in the future will need to find ways to circumvent this transcriptional suppression, for example using alternative germline specific promoters (Taxiarchi et al., 2019). Finally, more studies and methods are needed to systematically explore how population dynamics of released elements could be impacted by spontaneous genomic changes, such as transgene remobilization, done in a way that is technology-specific and relevant.

METHODS

Mosquito Rearing

Wild-type *An. gambiae* strain (G3) and transgenic mosquito strains were reared under standard conditions at 28°C and 80% relative humidity with access to fish food as larvae and 5% (wt/vol) glucose solution as adults. For egg production, young adult mosquitoes (3–5 days after emergence) were allowed to mate for at least 6 days and then fed on mice. Three days later, an egg bowl containing rearing water (dH_2O supplemented with 0.1% pure salt) was placed in the cage. One to 2 days after hatching, the larvae (L1 stage) were placed into rearing water containing trays. All animal work was conducted according to UK Home Office Regulations and approved under Home Office License PPL 70/8914.

Assaying Transgene Stability

PCRs were performed on selected transgenic and non-transgenic siblings that were screened twice during larval development for the DsRed phenotype. DsRed-positive and -negative individuals were examined by duplex and simplex PCR (GoTaq DNA polymerase, Promega). These PCR reactions amplified: 1) a fragment consisting of the wild-type genomic insertion site of the transgene i.e., the empty site that occurs in all individuals, regardless of whether they are transgenic or not, as a positive control; 2) a fragment of the internal DsRed marker; 3) fragments consisting of the known downstream (or upstream) flanking regions of the transgene (**Supplementary Figures S3, S4**). DNA was purified using the Qiagen Blood and Tissue kit.

Mosquito Whole Genome Sequencing and Read Mapping

Anopheles gambiae WGS reads from 81 individuals collected in Burkina Faso in 2012 were downloaded from the European Nucleotide Archive (Accession: PRJEB1670; **Supplementary Table S3**). WGS data from the G3 laboratory colony were downloaded from the SRA (Accession: PRJNA397539). Genomic DNA from 10 Ag(PMB)1 individuals was extracted using the Blood and Tissue Kit (Qiagen). For each sample, 100 ng of input gDNA was sheared using Covaris for a 350 bp insert size. Library preparation was performed using the Illumina TruSeq Nano kit. Each sample was tagged with a unique barcode, followed by three 2 × 150 bp High Output V2.5 paired-end sequencing runs on the Illumina NextSeq550 platform (PoloGGB, Sienna, Italy), obtaining an average of 265M reads per sample. WGS data from the Ag(PMB)1 have been deposited at NCBI SRA (Accession: PRJNA594202). Fastq reads were quality checked with FastQC (Andrews, 2015) and converted to fasta format. Reads were then mapped against the *vasa* driven piggyBac plasmid (Volohonsky et al., 2015) using blast blast-2.2.26/bin/blastall -i db. fa -d sample. fasta -p blastn -F ″m L″ -U T -e 1-e4 -a 40 -v 5 -b 40000 -K 40000. Only alignments with 98% identity over the entire read length were kept. Coverage was computed for each sample and normalized to the read depth of the most deeply sequenced sample using the following formula $X_i = X_i/(X_i/X_{max})$. To clarify plotting, read depth is reported every 10 bp.

Generation of Y-Chromosome Linked X-Shredder Transgenic Strains

The YpBac-β2-^{gfp}124L transgenic strain was generated as described in Galizi et al. (2014). Briefly, *An. gambiae* G3 embryos were injected with a mixture of 0.2 μg/μl of the pBac(3xP3-DsRed)β2-eGFP:I-*Ppo*I-124L plasmid and 0.4 μg/μl of helper plasmid containing a *vasa*-driven piggyBac transposase (Volohonsky et al., 2015). The hatched larvae were screened for transient expression of the DsRed marker and positives (~54%) crossed to wild-type mosquitoes. F1 progeny were analyzed for DsRed fluorescence and positives were crossed individually with wild-type mosquitoes to obtain transgenic lines. The transgene of one strain derived from a G0 male was identified that was transmitted exclusively to F1 sons, indicating Y-chromosome integration. The stain, now called YpBac-β2-^{gfp}124L was established and maintained by crossing to wild type females. The YattP- β2-^{gfp}124L strain was generated by co-injecting the pBac(3xP3-DsRed)β2-eGFP:I-*Ppo*I-124L construct with a *vasa*2-driven ΦC31 integrase helper plasmid (Volohonsky et al., 2015) into eggs of a strain containing a Y-chromosome AttP docking site (Bernardini et al., 2014). Crosses and screening were performed as above.

Sex Ratio and Fertility Assays

To assay adult sex ratio, transgenic males of each line were crossed to wild-type females. In all crosses, mosquitoes were allowed to mate for 3–5 days after the blood meal and gravid females were placed individually in oviposition cups. Larvae were reared to adulthood and sex was counted. The number of eggs laid as well as the number of larvae hatching were also counted, but only for the YattP-β2-^{gfp}124L to assay male fertility. The difference in sex bias among progeny of the Y-linked strains was tested independently to the expected 50% male ratio, using the chi-square test.

qRT-PCR Analysis

qRT-PCRs were performed on mosquito total RNA as described in Galizi et al. (2014). Briefly, 10 pairs of testes from each transgenic strain were pooled to constitute a biological replicate for total RNA and protein extraction using TRI reagent (Ambion). RNA was reverse-transcribed using Superscript II (Invitrogen) after TURBO DNA-free (Ambion) treatment following the manufacturer's instructions. Quantitative real-time–PCRs (qRT–PCR) analyses were performed on cDNA using the Fast SYBR-Green master mix on a StepOnePlus system (Applied Biosystems). Ribosomal protein Rpl19 gene was used for normalization. At least two independent biological replicates from independent crosses were subjected to duplicate technical assays. We used primers RPL19Fwd (5′-CCAACTCGCGAC AAAACATTC-3′), RPL19Rev (5′-ACCGGCTTCTTGATG ATCAGA-3′), eGFP-F (5′-CGGCGTGCAGTGCTTCA-3′), and eGFP-R (5′-CGGCGCGGGTCTTGT-3′). Internal normalization was done as in (Galizi et al., 2014) to the RPL19 ribosomal genes and normalized to expression from wild type testis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The accession numbers are: SRA, PRJNA594202, DQ236240.1, PRJEB1670, PRJNA397539. Other datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving animals were reviewed and approved by the UK Home Office with License PPL 70/8914.

AUTHOR CONTRIBUTIONS

PP, YA, and SF planned the study with help from all authors. RG, FB, and RH-K generated transgenic strains and characterized expression and transgene localization. RR performed individual PCR analysis confirming transgene location. DR, JA, PP, and MH designed and performed the computational experiments to assay for piggyBac presence in the mosquito genomes. PT contributed towards transgene translocation. PP, YA, and SF wrote the

manuscript. PP supervised the project with support from all authors.

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Ethical Considerations for Gene Drive: Challenges of Balancing Inclusion, Power and Perspectives

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Progress in gene-drive research has stimulated discussion and debate on ethical issues including community engagement and consent, policy and governance, and decision-making involved in development and deployment. Many organizations, academic institutions, foundations, and individual professionals have contributed to ensuring that these issues are considered prior to the application of gene-drive technology. Central topics include co-development of the technology with local stakeholders and communities and reducing asymmetry between developers and end-users. Important questions include with whom to conduct engagement and how to define community acceptance, develop capacity-building activities, and regulate this technology. Experts, academics, and funders have suggested that global frameworks, standards, and guidelines be developed to direct research in answering these important questions. Additionally, it has been suggested that ethical principles or commitments be established to further guide research practices. The challenging and interesting contradiction that we explore here is that the vast majority of these conversations transpire with little or no input from potential end-users or stakeholders who, we contend, should ultimately determine the fate of the technology in their communities. The question arises, whose concerns regarding marginalization, disempowerment, and inequity should be included in discussions and decisions concerning how inequities are perceived and how they may be addressed? At what stage will true co-development occur and how will opinions, perspectives and knowledge held by low-income country stakeholders be applied in determining answers to the questions regarding the ethics being debated on the academic stage? Our opinion is that the time is now.

Keywords: gene drive, ethics, engagement, co-development, responsible research, research guidelines

INTRODUCTION

The University of California Irvine Malaria Initiative (UCIMI), a not-for-profit research collaborative, has been actively involved in many of the discussions, workshops, and seminars addressing the application of gene-drive technology. The UCIMI mission is to contribute to malaria eradication through population modification of the African malaria vector mosquitoes, *Anopheles gambiae,* and *An. coluzzii,* rendering them incapable of transmitting malaria parasites to humans (Carballar-Lejarazú et al., 2020). Our perspectives here represent those of a research program that has developed gene-drive systems for public health application using a relationship-based approach (Kormos et al., 2020).

A simple internet search for "gene drive" will demonstrate the large number of perspectives surrounding this technology and the questions of whether and when to apply it. Numerous publications explore these questions, and many also offer conceptual frameworks, guidance, or ethical considerations for developers of gene drive (Lavery et al., 2010; Ramsey et al., 2014; WHO Special Programme for Research and Training in Tropical Diseases, 2014; National Academies of Sciences Engineering and Medicine, 2016; Emerson et al., 2017; Roberts et al., 2017; AUDA-NEPAD, 2018; Collins, 2018; James et al., 2018; Brossard et al., 2019; Thizy et al., 2019; Deplazes-Zemp et al., 2020; World Health Organization, 2020; Annas et al., 2021; World Health Organization, 2021). These guidelines are developed for broad application, to be adapted to local environments. This alone poses challenges associated with political, social, regulatory and environment complexities and differences from one place to another. We believe that guidance should be developed on a case-by-case basis for application in alignment with the relationship-based model (Kormos et al., 2020). However, we do not focus here on this point, rather we argue that in the development of guidance, whether developed locally or globally, it is critically important to consider whose perspectives and values are included in the development process.

In addition to publications, there are institutions and organizations exploring these questions through workshops and webinars; gathering experts and academics together to discuss perspectives and ideas on the topic. For example, in 2021 the Gene Convene Global Collaborative hosted a series of virtual panel discussions entitled "Considering the case of Gene Drive Technologies Through Social Science Theories on Stakeholder Engagement" and a second session under the heading, "Unsettled Ethical Issues in Gene Drive Research."

With few exceptions, the primary authors, organizations, and institutions engaged in these discussions, like gene-drive developers themselves, are not located in areas where the technology is being proposed for use to address public health concerns. The voices and values represented are largely those of individuals who are not living in communities that will be directly impacted by the application of this technology. Additionally, many of these academic experts are not working directly with practitioners in the field. This is the interesting challenge we would like to explore further here; the increasing divergence between academic and theoretical recommendations and those of the practitioners engaged directly with stakeholders and community members. This difference in understanding presents several specific areas of concern: 1) Perspectives - the voices that are front-and-center in these discussions do not necessarily represent the perspectives and values of those who share the greatest risk/benefit of the application of the technology, 2) Inclusion - while emphasizing the importance of co-development, much of the proposed guidance and recommendations is not co-developed with stakeholders, and 3) Power—the current system of decision-making and knowledge production enhances the imbalance of power and restricts illumination of important local perspectives and insights.

The uncertainty of outcomes associated with the application/testing of this novel technology has created an expansive niche for academic exploration of the important ethical and moral questions associated with it. While these are critically important issues to consider, it is just as important, and we would argue necessary, to evaluate whose values and voices ought to be driving the answers to these questions.

PERSPECTIVES: VOICES AND VALUES INFLUENCING GENE DRIVE RESEARCH

Proposed guidelines, principles, and commitments for gene-drive research present recommendations for ethically-responsible development and deployment practices for researchers (Lavery et al., 2010; Ramsey et al., 2014; WHO Special Programme for Research and Training in Tropical Diseases, 2014; National Academies of Sciences Engineering and Medicine, 2016; Emerson et al., 2017; Roberts et al., 2017; AUDA-NEPAD, 2018; Collins, 2018; James et al., 2018; Brossard et al., 2019; Thizy et al., 2019; Deplazes-Zemp et al., 2020; Long et al., 2020; World Health Organization, 2020; Annas et al., 2021; World Health Organization, 2021). It is critically important that application of the technology follow practices of social responsibility, transparency, accountability, and compliance with local governance and regulatory infrastructure. If we are to create guidance frameworks intended for widespread application, it also is important to consider whose perspectives helped develop the principles and guidelines meant to ensure these practices.

A quick review of the authors and associated institutions and organizations of most published recommendations reveal that this work is led largely by academics and experts who are not engaged directly in research and engagement activities at proposed field sites.

The same situation pertains to workshops and symposia designed to bring diverse perspectives together to discuss important questions and challenges surrounding gene-drive research, such as whom, when and how to conduct engagement, how to define and determine community consent/acceptance, and what ethical principles should guide responsible research. These broad and complex topics are typically led and facilitated by academic and professional experts who offer important insights in these areas. While discussions lead to an expanded list of questions and considerations for gene-drive research, too often, voices and values represented in these venues are not representative of those who are regularly engaged with communities and stakeholders in the field, or directly involved in managing and conducting research at field

sites. In addition to providing important insights about diverse, often complex, community perspectives and values, stakeholders and community leaders also offer understanding about local governance and regulation that will likely impact and influence how such guidance is implemented. Individuals who are closest to the work in the field and grappling actively with these challenges are under-represented. Ethically-responsible development of this technology is especially important for public health applications where guarantee of the communities' best interests, and respect for their ideology and values, need to be factored into the principles guiding the work. Collaborations with existing ethics committees, social scientists, public health professionals, and biosafety/biotechnology regulators from field sites offer essential perspectives to help guide best practices and responsible development of the technology. It has been suggested previously that regulators from field sites should be involved directly in the facilitation and adaptation of essential guidelines and frameworks for the technology (AUDA-NEPAD, 2018).

If we are to create guidance and recommendations for widespread implementation and to influence research practice and ensure ethically-responsible development, is it not essential to involve the perspectives of those most directly involved? Involvement requires intentional planning and communication with developers and their collaborators at field-sites to build a connection with appropriate stakeholders. It necessitates rigorous reciprocal engagement between developers and stakeholders for exchange of knowledge and technical capacity building to support advanced understanding of genetic principles and molecular biology that apply to the technology. It requires stakeholder invitation to participate in meetings, workshops, and in the collaborative writing process. It calls for collaboration with groups who may not have the same experience and knowledge but whose opinions need to be taken into account so that they are part of the solution. Without the collaboration of field-site stakeholders and field practitioners, we risk creating recommendations lacking representation of the voices and values of entire groups for whom the recommendations are designed in large part to protect and support.

INCLUSION: ACTIVATING THE CONCEPT OF CO-DEVELOPMENT

Co-develop is defined as "to develop (something) by working with one or more others to develop (something) jointly" (Merriam-Webster, 2021). Co-development is articulated as an important, if not essential, element in the development and application of gene drive technology(Target Malaria, 2017; Hartley et al., 2019; Thizy et al., 2019; Kormos et al., 2020; World Health Organization, 2021) The recently-published second edition (2021) of the WHO Guidance for Testing Genetically Modified Mosquitoes, places importance on "a co-development approach that emphasizes authentic partnership and knowledge engagement" for community engagement and development of the technology in general (World Health Organization, 2021). While numerous publications emphasize the importance of co-development, the process of their creation does not regularly apply the practice. We believe that it is essential to include stakeholders and practitioners from field sites in the work of developing guidance and commitments for the development and application of the technology, particularly considering that these are the individuals with whom we should be partnering to ensure a "co-development approach" is applied.

Sharing of knowledge and research and investing in relationships of trust and collaboration are essential in the process of co-developing a shared set of goals and a research pathway to reach those goals. Co-development requires trust (Athaide et al., 2003; Nielsen, 2004; Bidault and Castello, 2019). Trust between research programs and stakeholders and communities. Some recommendations call for a "neutral" third-party facilitator for engagement to avoid potential conflicts of interest, ensure ethically responsible research practice, and maintain balance of power (Kofler et al., 2018). We argue that these recommendations would restrict, if not eliminate, opportunities for true co-development, knowledge sharing and bi-directional communication essential to build trust between a research project and relevant communities. Co-development requires trust and knowledge sharing between these groups; knowledge from communities is essential to inform project practice, principles, target goals, and to establish a balance of power. Recommendations for engagement, and navigation of complex challenges associated with decision-making, and conflicts of interest should be developed at a minimum in consultation with multidisciplinary local experts and those with knowledge and experience in areas where they may be applied.

How do we develop guidelines for best practice and recommendations for ethical engagement and inclusion without partnering with those who are closest to the values and priorities of communities and stakeholder groups that will be affected most by the technology? True belief in co-development calls for co-development of guidelines, which require collaborative work and "authentic partnership" (participants share in the conceptualization, development, and sharing/publication) with a much broader group of stakeholders. This practice begins with an acknowledgement and commitment from the global gene-drive research community to apply co-development practice in the evolution of published guidelines and recommendations, and to place value and trust in the critical perspectives and values of these stakeholder groups. It is essential, for successful co-development of guidelines, to first engage in rigorous knowledge exchange to build capacity of participating groups. This concept of mutual learning and knowledge exchange is well elaborated in the NASEM publication *Genes Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values* (National Academies of Sciences Engineering and Medicine, 2016). The African Union, New Partnership for Africa's Development (AUDA-NEPAD) and the Pan-African Mosquito Control Association have initiated important first steps in essential knowledge engagement with potential end-user stakeholders (AUDA-NEPAD, 2018). NEPAD has organized meetings and workshops to discuss gene drives and their potential uses with African stakeholders, and PAMCA (Pan-Africa Mosquito Control Association, 2021), in partnership with Target Malaria (a program developing gene drives for suppressing mosquito populations), has provided training courses on gene drives targeted toward participants that include researchers,

policymakers, health professionals, and graduate students. The expansion of knowledge engagement is an essential first step in the facilitation of meaningful co-development of guidelines for potential application.

Without making these efforts, the recommended practices and considerations remain largely reflective of the specific values and ethical concerns of academic experts and institutions, maintaining an imbalance of power and influence.

POWER: SHIFTING KNOWLEDGE-PRODUCTION AND DECISION-MAKING TO LOCAL EXPERTS

The subject of power dynamics in gene-drive research has been of particular interest given that the development of the technology is occurring largely in high-income countries (HIC) for deployment in low-to-middle-income countries (LMIC). Large social and economic inequalities between the two, as well as perceptions of historical injustices, are likely to influence the way that knowledge is produced and foreign investment is perceived (National Academies of Sciences Engineering and Medicine, 2016; Rudenko et al., 2018; Kofler and Taitingfong, 2020). Some authors have pointed to inequity and history as potential threats to co-development and the creation of fair and equal partnerships between developers, communities, and governing bodies (Athaide et al., 2003; Nielsen, 20042004; Target Malaria, 2017; Kofler et al., 2018; Rudenko et al., 2018; Bidault and Castello, 2019; Hartley et al., 2019; Kofler and Taitingfong, 2020; Long et al., 2020; Ledingham and Hartley, 2021; Merriam-Webster, 2021). Proposed guidance, frameworks, and webinar discussions offer ways to acknowledge and counterbalance these dynamics and achieve engagement, collaboration, and shared decision making (Matenga et al., 2019; Thizy et al., 2019; Turnhout et al., 2020). As we point out, HIC authors and experts are leading these proposed solutions to inequities in power. Additionally, the specific HIC recommendations for gene-drive research places developers in a position that may require demanding specific criteria or processes that disrespect or disregard local governance and values. It is for these reasons that the inclusion of local perspectives, particularly those of regulators and governing bodies, be integral in the development of proposed guidance/frameworks. An understanding of local governance structures, and how and when they should be involved as partners or as providers of guidance regarding policy and legal frameworks, would assist in the development of guidance that maintains respect for local values. It is important to consider how knowledge is produced and shared, and what information is valued and by whom. A suggested solution to balancing this tension is to position local experts in the lead in knowledge production and decision making (De Graeff et al., 2021). It is legitimate to consider that people aspire to be more than mere spectators in the battles that are being fought in their name, or for their benefit, otherwise it would represent a kind of paternalism or condescension towards them. The best way to achieve authentic partnership with people in any initiative is to involve them in the entire process, under risk of generating skepticism and rejection.

In practice, how do local practitioners and experts take a lead in complex, and competitive practice dominated by the HIC academy? Our proposed first step is their inclusion in the conversations that often lead up to publication of guidance and recommendations. It is up to the current influencers (funders, institutions, and academic experts) in the gene-drive community to put into action the practice of inclusion, co-development, and true engagement that we have articulated as a priority.

CONCLUSION

Gene-drive technologies developed for public health applications are complex and present a set of challenging issues that should be explored and considered thoughtfully prior to their application. The uncertainty of outcome of the technology, and the associated risks and benefits, raise important ethical questions and concerns for discussion and exploration. If we are to approach this novel technology in the spirit of true co-development and with determined effort to be inclusive, minimize inequities and imbalances in power, and illuminate the voices and values of those who will be most affected by the application, we need to change our approach to the work. It is essential to include field-site practitioners, stakeholders and community leaders in the academic conversations and debates surrounding these subjects. A new value needs to be placed on reaching these voices and creating a space for sharing their knowledge and prioritizing their perspectives. Without this, the guidelines and recommendations for gene drive presented by the academic community and HIC funders and institutions will fail to meet the ethical goals and commitments they want to achieve.

> "Whatever you do for me but without me, you do against me"
>
> Mahatma Gandhi

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

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Introgression of the *Aedes aegypti* Red-Eye Genetic Sexing Strains Into Different Genomic Backgrounds for Sterile Insect Technique Applications

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Aedes aegypti is an invasive mosquito species and major vector of human arboviruses. A wide variety of control methods have been employed to combat mosquito populations. One of them is the sterile insect technique (SIT) that has recently attracted considerable research efforts due to its proven record of success and the absence of harmful environmental footprints. The efficiency and cost-effectiveness of SIT is significantly enhanced by male-only releases. For mosquito SIT, male-only releases are ideally needed since females bite, blood-feed and transmit the pathogens. *Ae. aegypti* genetic sexing strains (GSS) have recently become available and are based on eye colour mutations that were chosen as selectable markers. These genetic sexing strains were developed through classical genetics and it was shown to be subjected to genetic recombination, a phenomenon that is not suppressed in males as is the case in many Diptera. The genetic stability of these GSS was strengthened by the induction and isolation of radiation-induced inversions. In this study, we used the red eye mutation and the inversion Inv35 line of the *Ae. aegypti* red-eye GSS s and introgressed them in six different genomic backgrounds to develop GSS with the respective local genomic backgrounds. Our goal was to assess whether the recombination frequencies in the strains with and without the inversion are affected by the different genomic backgrounds. In all cases the recombination events were suppressed in all Inv35 GSS strains, thus indicating that the genomic background does not negatively affect the inversion result. Absence of any effect that could be ascribed to genetic differences, enables the introgression of the key elements of the GSS into the local genomic background prior to release to the target areas. Maintaining the local background increases the chances for successful matings between released males and wild females and addresses potential regulatory concerns regarding biosafety and biosecurity.

Keywords: area wide integrated pest management, insect pest control, vector control, mosquitoes, yellow fever mosquito

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INTRODUCTION

Arthropod-borne viruses or "arboviruses" transmitted by *Aedes* spp. mosquitoes are accountable for the emergence of human epidemic diseases across the globe (Weaver and Reisen 2010; Lucey and Gostin 2016; Siraj et al., 2017; Wilder-Smith et al., 2017). Zika, dengue, yellow fever, and chikungunya viruses infect humans by the bite of an infected *Aedes aegypti* L. (Diptera: Culicidae) mosquito and result in a diverse array of clinical symptoms and implications ranging from systemic febrile illnesses to neurological or cerebrovascular diseases and death (Bhatt et al., 2013; Beckham and Tyler 2015; Paixão et al., 2018). *Ae. aegypti* has successfully spread in tropic and subtropic zones worldwide (Kraemer et al., 2015). It is daylight-active, thrives in urban and peri-urban areas, feeds exclusively on human blood multiple times during a gonotrophic cycle, and shows high susceptibility to arboviruses (Scott and Takken 2012; Wilder-Smith et al., 2017; Ryan et al., 2019). Its ability to breed in human-made breeding settings facilitates the increase of the vector's population and fuels the spread of the vector-borne diseases. Urbanization of rural areas, increase of travelling activities, globalization, and climate change accelerate the invasion potential of *Ae. aegypti* and enhance the viral transmission (Wilder-Smith and Gubler 2008; Bhatt et al., 2013; Struchiner et al., 2015; Ryan et al., 2019; Iwamura et al., 2020).

The lack of effective drugs and vaccines against these arboviruses (apart from the yellow fever vaccine) has shifted the spotlight on the vector population control methods (Achee et al., 2015; Lees et al., 2015; Bourtzis et al., 2016; Flores and O'Neill 2018). Current efforts rely on insecticide applications and elimination of breeding sites; however, these methods have been proved both unsustainable and inefficient. The development of insecticide resistance, the rapid expansion of *Ae. aegypti* populations in urban areas and the inadequate control of the cryptic breeding sites led scientists and communities to pursue environmentally-friendly approaches that would control efficiently the vector populations without compromising sustainability (Lima et al., 2011; Achee et al., 2015; Louis et al., 2016; Moyes et al., 2017).

During the recent years, numerous genetically based approaches have been developed aiming either to modify vector populations (i.e., rendering them resistant in pathogen transmission) or to suppress them below the threshold required for disease transmission (Harris et al., 2012; O'Connor et al., 2012; Alphey et al., 2013; Bellini et al., 2013; Carvalho et al., 2015; Mains et al., 2016; Kittayapong et al., 2018; Kyrou et al., 2018; Kandul et al., 2019; Kittayapong et al., 2019; Zheng et al., 2019; Crawford et al., 2020). Some of the population suppression approaches, including the sterile insect technique (SIT), have been tested in the field with encouraging results (O'Connor et al., 2012; Bourtzis et al., 2014; Bourtzis et al., 2016; Kittayapong et al., 2018; Kandul et al., 2019; Kittayapong et al., 2019; Zheng et al., 2019; Crawford et al., 2020). The SIT which relies on the mass production and release of sterile males, has historically been applied for the control and eradication of insect pest populations (Bushland et al., 1955; Knipling 1955; Klassen et al., 2021). When considering the SIT as part of a mosquito control project, one of the greatest challenges to be addressed is the sex separation and elimination of females (Papathanos et al., 2009; Gilles et al., 2014). Unlike agricultural pests where either bisexual release is the only feasible approach or the accidental release of few females is not considered a major concern, in mosquitoes, release of both males and females is a no go, since adult females create biting nuisance, and are potential disease vectors. Thus, an adequate and robust sex separation system that will reliably separate male and female mosquitoes at a large scale is of critical importance for the implementation of a SIT program (Gilles et al., 2014; Papathanos et al., 2018). In *Ae. aegypti*, sex separation is currently based on the inherent characteristics of the species, i.e., the size dimorphism between male and female pupae and male-specific body parts of adults including genitalia and antennae (Focks 1980; Gunathilaka et al., 2019; Crawford et al., 2020). This approach is rearing-dependent, prone to errors, labor-intensive and appropriate for small-scale operations. Although novel and (semi)-automated methods have been developed, the critical need of a genetic sexing strain (GSS) for *Ae. aegypti* rises as the ideal sex separation method particularly if males and females could potentially be separated at early developmental stages (Gilles et al., 2014; Papathanos et al., 2018). Developing a GSS using classical genetics typically requires a selectable marker (visually detectable or conditionally lethal) and the linkage of the wild type allele of this marker to the Y chromosome or to the sex-determining genetic locus (Franz et al., 2021).

Aedes species have homomorphic sex chromosomes and their maleness is defined by a dominant male-determining locus (M locus) of chromosome 1 (Craig and Hickey 1967; Newton et al., 1974; Hall et al., 2015; Aryan et al., 2020; Liu et al., 2020). *Ae. aegypti* males are heterogametic (Mm) while the females are homogametic (mm) for the M-locus (Timoshevskiy et al., 2013). The competence of the selectable marker will in turn determine the robustness of the GSS and in *Ae. aegypti* the ideal marker would reside on chromosome 1, closely linked to the M-locus. In such a strain, male mosquitoes would be heterozygotes and express the "wild-type" phenotype while females would be homozygous for the recessive alleles of the selectable marker expressing the mutated phenotype (Franz et al., 2021). Promising markers that could be used for *Ae. aegypti* GSS development are related to eye colour (Red-eye (*re*) and White-eye (*w*) markers) which are located on chromosome 1 linked to the M-locus and they are fully penetrant and expressive (Bhalla and Craig 1970; Munstermann and Craig 1979). Both markers have been used by our group for the construction of two *Ae. aegypti* GSS, in which males have black eyes and females have either red or white colour eyes (Koskinioti et al., 2020). The *re* and *w* mutant lines were crossed with the wild-type "BRA" strain collected from Brazil and the Red-eye GSS and White-eye GSS were developed. Quality control of both GSSs evidenced no significant differences regarding sex ratio and immature development duration of both sexes. The Red-eye GSS showed outstanding productivity compared to the White-eye GSS and significantly elevated lifespan and flight ability compared to the wild type "BRA" strain (Koskinioti et al., 2020).

The stability of a GSS, in particular under the demanding mass-rearing conditions, is a pivotal factor for its successful implementation in operational population suppression programmes. Instability during mass-rearing conditions is mainly

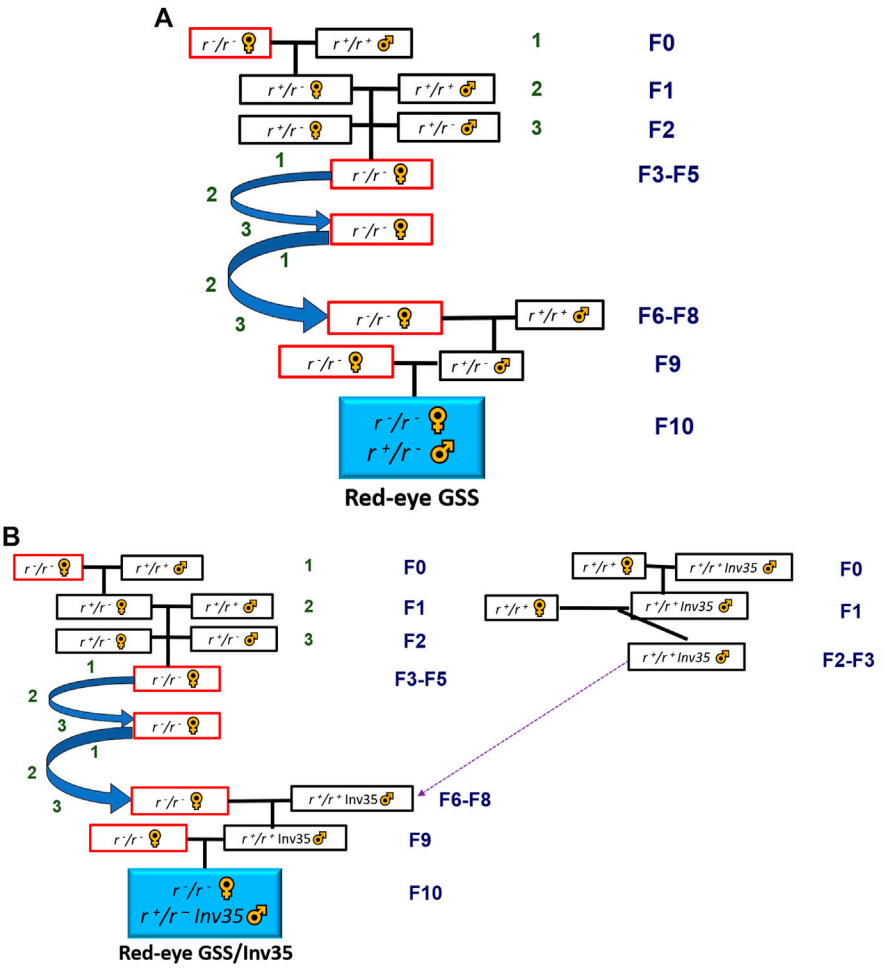

FIGURE 1 | (A) Crossing scheme for the introgression of the red eye mutation into a local genomic background, **(B)** Crossing scheme for the introgression of the red eye mutation and the inversion 35 into a local genomic background. Both schemes are simplified and not all genotypes retrieved after each cross are depicted. See **Supplementary Methods S1 and S2** for detailed analysis.

attributed to genetic recombination events. Unlike other Diptera, in *Aedes* mosquitoes, recombination events occur in males almost as frequently as in females, and these events can compromise the GSS stability and lead in breakdown of the GSS due to accumulation of recombinants. Incorporation of recombination-suppressing factors, such as inversions, can improve the stability of a GSS (Franzet al. 2021; Gilles et al., 2014; Zacharopoulou et al., 2017). In *Ae. aegypti* induction of inversions has been shown to suppress recombination between the M locus and morphological markers of chromosome 1 (Bhalla 1973). Using irradiation our group induced inversions in *Ae. aegypti* and showed that irradiation frequency can be suppressed between *re* and the M locus (line 35), while at least two lines in which recombination is suppressed between *w* and the M locus (lines 5 and 35) were identified (Augustinos et al., 2020). Inversion line 35 was incorporated in the Red-eye GSS and White-eye GSS by crossing wild-type males having the recombination suppressor (from the Inv35 line) with females from the two GSSs. Recombination frequencies were measured for consecutive generations under filtered and non-filtered conditions, i.e., removal or not of recombinant progeny from each generation, and recombination was consistently reduced for both strains (Koskinioti et al., 2020).

GSSs may still face issues when released in the field that could lead in performance reduction. The genomic background has been shown to be a driving factor when it comes to mosquito performance. Among others, variation in vector competence, reproductive incompatibility, effects on fitness traits and differences in the reproductive effects of *Wolbachia* infections have been shown to stem from variations in the genomic background of mosquito populations (Bennett et al., 2002; Menge et al., 2005; Axford et al., 2016; Dickson et al., 2016; Campbell et al., 2017; Carvalho et al., 2020; Enkerlin 2021). The success of sterile mosquito releases relies massively on the mating performance of the released males. However local mosquito populations might vary significantly in terms of ecology, biology, and behavior and this could in turn lead to mating barriers which would compromise the efficiency of a SIT programme (Krafsur and Ouma 2021). These barriers can be overcome by developing mosquito GSS that will be integrated

into the local genomic background of the release area. In this study the *A. aegypti* red eye mutation and the inversion Inv35, the latter developed previously in the Insect Pest Control Laboratory (IPCL, Seibersdorf, Austria), were introgressed in populations originating from different geographic areas to develop Red-eye GSS and Red-eye GSS/Inv35 strains with local genomic backgrounds and their genetic stability was assessed for several generations (Augustinos et al., 2020; Koskinioti et al., 2020).

MATERIALS AND METHODS

Ae. aegypti Strains and Rearing Conditions

The Rexvillle Red Eye strain, which is homozygous for the recessive *re* allele, was used in the present study and was kindly provided by Dr. Margareth Capurro at the Department of Parasitology, University of Sao Paulo, Brazil. In the *re* strain all individuals have red eye color which is evident throughout all developmental stages and it darkens as adults age. Six wild type *Ae. aegypti* strains originated from Brazil (BRA), Indonesia (IDN), Mexico (MEX), Singapore (SGP), Sri Lanka (LKA), and Thailand (THA) were used for the introgression crosses described below and checked for their recombination rates. The *Ae. aegypti* inversion line 35 (Inv35) (Augustinos et al., 2020) was used to incorporate the inversion in all six genomic backgrounds. In all wild-type strains the eye color is dark brown/black and remains stable at all developmental stages. All strains were maintained in the insectary of the Insect Pest Control Laboratory (Joint FAO/IAEA Centre, Seibersdorf, Austria) at 27 ± 1°C, 80% relative humidity and a 12/12 h day/night photoperiod.

Adult mosquitoes were kept in standard (30 × 30 × 30 cm) insect plastic rearing cages (BugDorm-41,515 insect cage) and a 10% sucrose solution was constantly provided. Female mosquitoes were blood-fed with porcine blood twice per week. The blood used was collected in Vienna, Austria during routine slaughtering of pigs in a nationally authorized abattoir, conducted at the highest possible standards strictly following EU laws and regulations. Egg collections were initiated 72 h after the last blood feeding using moistened oviposition papers (white germination paper, Sartorius Stedium Biotech, Austria).

Crosses

Development of Red Eye-GSS Strains in Local Genomic Backgrounds

Females of the Red Eye strain and males from IDN, SGP and LKA populations were used to initiate the three introgression crosses while the respective crosses with males from BRA, MEX and THA populations are reported in the study of Chen et al. (2021) (**Figure 1A**).

The introgression of Inv35 in the local genomic backgrounds was initiated independently and it was continued until a semi-introgressed inversion line had been acquired. At that stage, partially Introgressed Inv35 males were crossed with highly introgressed Red Eye females to create a Red-GSS with Inv35 in a local genomic background (**Figure 1B**). The genomic backgrounds of the introgressed Red Eye females were from BRA, IDN, MEX, SGP, LKA and THA populations. In all cases, fifty females and twenty males were crossed in every generation in a 15 × 15 × 15 cm rearing cage (BugDorm-4M1515). The detailed introgression protocols are provided in the **Supplementary Materials S1 and S2**.

Estimation of Recombination Rate

The recombination rate was estimated for all the newly established Red-eye GSS and Red-eye GSS/Inv35. All progeny were screened in every generation and recombinants (males with red eyes and females with black eyes) were recorded and subsequently discarded. At least six generations per strain were screened. Black eye males and red eye females were used to set up the new cages. A minimum number of 1,000 individuals were used to set up the new cages.

Data Analysis

All statistical analyses were performed using R version 4.0.5 (R Core Team, 2021). The recombination rates between the strains with and without inversion of the same origin and among the different origins represent proportional data and therefore, they were analyzed using a GLM-binomial family and a logit link function (Dunn and Smyth, 2018). In case overdispersion was detected, a Quasi-Binomial model with a logit link function was applied (Demétrio et al., 2014). Analysis of deviance was performed with a Chi-squared test for GLM-Binomial models and with a F-test for GLM-Quasi-Binomial models (Nelder and Wedderburn, 1972). Residuals of the models were checked for normality and homogeneity of variance. Goodness-of-fit of the models was visually inspected with half-normal plots with simulation envelopes (Moral et al., 2017). Emmeans package was used for the pairwise comparisons of the fitted model estimates (Searle et al., 1980).

RESULTS AND DISCUSSION

The *Ae. aegypti* Red-eye GSS has been developed through classical genetics and is based on the *re* morphological marker that has been mapped to chromosome I (Koskinioti et al., 2020). The red eye mutation presents full penetrance and expressivity and the red eye color is evident throughout all developmental stages. Estimation of the recombination frequency between *re* and M locus confirmed that *re* is a recessive, sex-linked gene. Recombination events in *Aedes* species occur both in males and females and, in the case of a GSS under mass-rearing conditions, they can eventually lead in reduced genetic stability and colony collapse (Augustinos et al., 2017; Franz et al., 2021). Elements that suppress recombination between the M locus and the marker are therefore required to be incorporated in the GSS. In the study by Augustinos et al. (2020) an inversion (Inv35) was induced through irradiation aiming to suppress recombination between *re* and the M locus. Indeed, the recombination frequency was significantly suppressed, and the inversion was incorporated in the Red-eye GSS thus creating the Red-eye GSS/Inv35. These two strains were screened for numerous generations and results demonstrated significantly decreased recombination in the

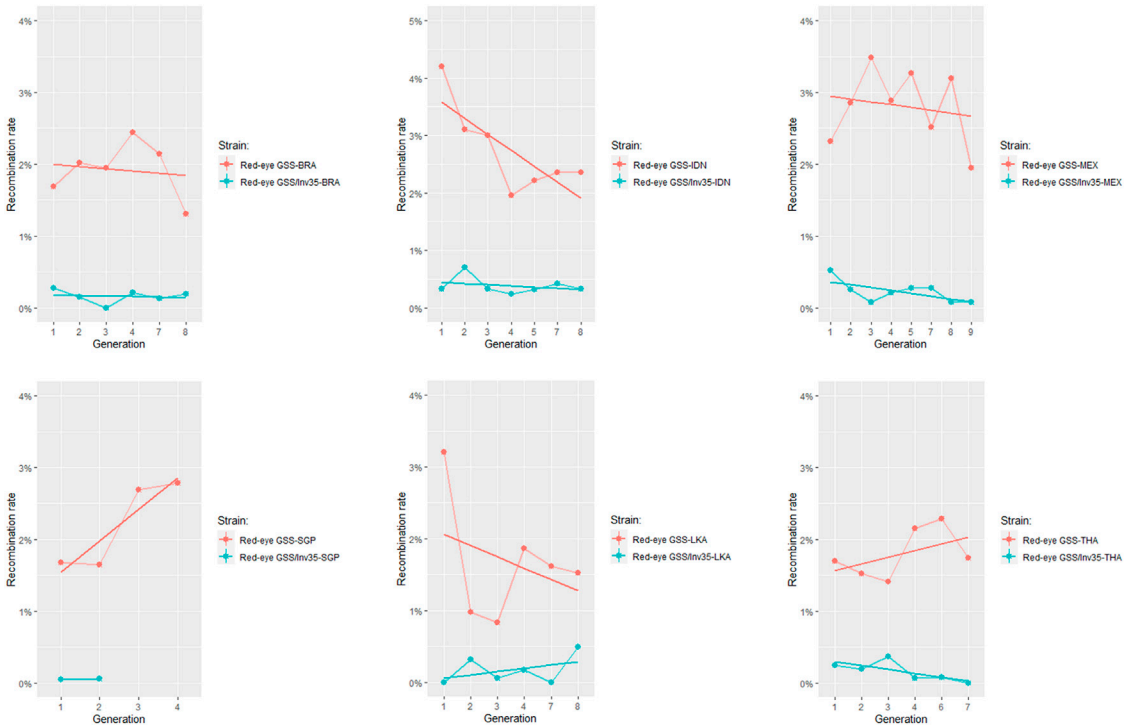

FIGURE 2 | Recombination rates of the Red-eye GSS and Red-eye GSS/Inv35, after their introgression in six local genomic backgrounds. Recombinant males and females were recorded in each generation and results were analyzed with a GLM (binomial family). In all genomic backgrounds, the strain incorporating the inversion had significantly lower recombination rates compared to the respective strains without the inversion. The straight line represents the fitted linear model.

Red-eye GSS/Inv35 compared to the original strain (Koskinioti et al., 2020).

Variability in recombination frequencies can be attributed, among other factors, to genomic differences, with chromosomal rearrangements being the most likely reason (Dickson et al., 2016). In the present study, we received six *Ae. aegypti* populations from countries that could be possible target areas of a future operational SIT programme. The red eye mutation line and the inversion Inv35 were introgressed into the six genomic backgrounds, following a crossing scheme that lasted for eleven generations. Our goal was to assess whether the novel genomic background would affect the recombination frequencies that had

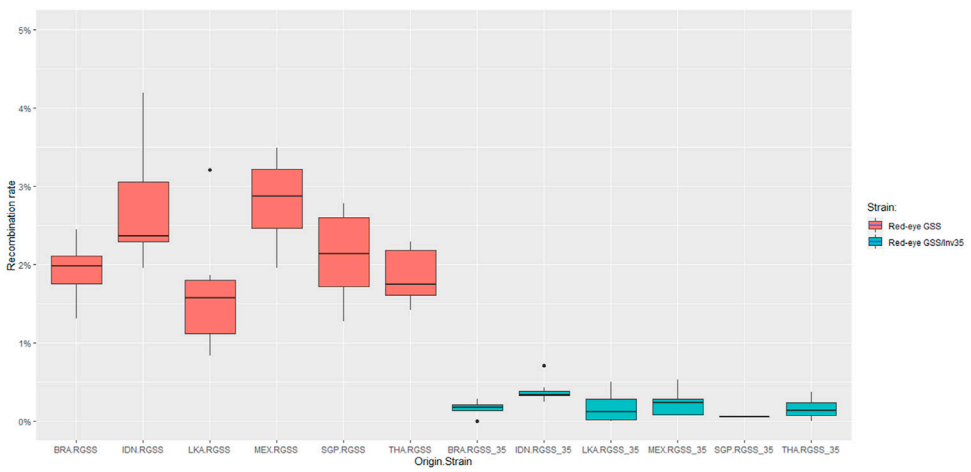

FIGURE 3 | Recombination rates of the Red-eye GSS and Red-eye GSS/Inv35 throughout the course of generations. Generations were used as replicates. No significant effect was detected among the Red-eye GSS/Inv35 strains, indicating that Inv35 suppresses recombination irrespectively of the genomic background.

been estimated in the original GSS. As soon as the introgression crossing scheme was completed and the twelve new strains had been established, the recombination frequencies were evaluated for all strains. A total of 110,799 mosquitoes from 73 generations of 12 strains were screened and recombination frequencies were recorded (**Supplementary Materials S3 and S4**).

The recombination frequency was estimated for each genomic background individually and results indicated that the Red-eye GSS/Inv35 presented significantly lower recombination rates compared to the Red-eye GSS throughout the course of generations (**Figure 2**). Except for Singapore for which data availability is limited, the strains with the inversion were more stable and with significantly lower recombination rates compared to the ones without (**Supplementary Material S5**). Recombination frequencies were analyzed to check for any possible effect of the genomic background, using data from different generations as replicates. In all genomic backgrounds the recombination rates were significantly lower for the Red-eye GSS/Inv35 ($F = 51.375$, $df = 11$, $p < 2.2e\text{-}16$), thus indicating that the effect of inversion is evident in all genomic backgrounds (**Figure 3** and **Supplementary Material S6**). Assessment of the recombination frequencies among the six Red-Eye GSS/Inv35 strains showed no statistically significant differences, thus suggesting that the inversion suppresses recombination similarly, irrespective of the genomic background (**Supplementary Material S6**). Interestingly, the pairwise comparisons of the six Red-Eye GSS strains showed an effect of the genomic background on the recombination rates. Red-eye GSS-BRA was shown to be significantly different from the MEX ($z = -3.495$, $p = 0.0005$) and IDN ($z = -3.035$, $p = 0.0024$) strains, while the same was also true for the IDN-LKA ($z = 3.422$, $p = 0.0006$), IDN-THA ($z = 2.855$, $p = 0.0043$), MEX-LKA ($z = -3.889$, $p = 0.0001$), and MEX-THA ($z = 3.353$, $p = 0.0008$) Red-Eye GSS comparisons. However, in some Red-eye GSS strains *per se* the recombination frequencies varied among generations. Results for Indonesia, Mexico, and Sri Lanka showed that there was a statistically significant difference among the tested generations (**Supplementary Material S7**) which could be attributed to factors such as age, sex and temperature (Augustinos et al., 2020). No variation was detected for the Red-eye GSS from Brazil, Singapore and Thailand. The same conclusion was also reached for the Red-eye GSS/Inv35 strains (**Supplementary Material S7**). The recombination rates were stable through the course of generations per strain and no statistically significant differences were detected for all the genomic backgrounds which confirmed the robustness and effectiveness of the inversion in suppressing recombination regardless of genomic background and generation. This clearly indicated that the genomic background did not negatively affect the genetic stability of the strains and confirmed the robustness and effectiveness of the inversion in suppressing recombination regardless of genomic background and generation. The Red-eye GSS/Inv35-SGP is the only strain for which availability of data is limited and therefore more generations are required to reach a safe conclusion.

The results of the present study are encouraging, in respect to the genetic stability of Red-eye GSS/Inv35 developed in local genomic backgrounds. However, the biological quality of the newly established GSS needs to be assessed first under laboratory and later in field conditions (Carvalho et al., 2020; Koskinioti et al., 2020). The genomic differences might be proved detrimental to important fitness traits as has been shown in both fruit flies and mosquitoes (Meza et al., 2011; Facchinelli et al., 2013; Rempoulakis et al., 2016; Ramírez-Santos et al., 2017; Ramírez-Santos et al., 2017; Carvalho et al., 2020). An in-depth quality control analysis that will assess important parameters like fecundity, fertility, longevity, flight ability, male mating competitiveness and response to irradiation, prior to upscaling and releasing in the field.

The release of a mosquito GSS as part of an operational SIT programme is ruled in most cases by concerns regarding the biosafety and biosecurity of the released strain, as well as by uncertainties related to the performance of the strain in the wild. A mosquito GSS has been developed and reared in laboratory conditions for several generations and carries its own genomic background. Decision-making bodies could reject the release of a GSS in an area based on the notion that breeding of human disease vectors bearing different genomic backgrounds could result in previously undetected risks related to humans and the environment. To address these issues, it is advisable to use either a local strain or to integrate the mosquito strains into the local genomic background prior to release. That way the potential effects associated with mating incompatibility are minimized and the chances for increased male mating competitiveness are raised since the released males and the wild females will share the same genomic background. In addition, maintaining the local genomic background can resolve any regulatory issues posed by the countries, reaffirm the biosecurity and biosafety of the released strain, and enhance the public acceptance towards the SIT programmes.

The recent discovery of the gene responsible for the red eye phenotype in *Aedes aegypti*, namely *cardinal*, opens the way for a faster and easier transfer of the sexing characters of the red eye GSS in local genomic background and will thus avoid the long (10–11 generations) and tedious genetic crosses described in this manuscript (Chen et al., 2021). This can be achieved by using CRISPR/Cas9 targeted mutagenesis of the *cardinal* gene of the local population to *de novo* develop a red eye mutant line. Next step would be to perform two simple genetic crosses as the ones described for the original construction of the red-eye GSS (Koskinioti et al., 2020). First, mutant females should be crossed with wild type males and second, F1 males should be backcrossed with mutant females to establish a genetic sexing strain with local genomic background.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AA: conceived, designed, and performed the experiments, interpreted the data, drafted the first draft of the manuscript, and contributed to the preparation of the final version. KN: analyzed the data and wrote the manuscript. LD, MM-u-H, DC performed experiments. KB: conceived and supervised the study, designed the experiments, interpreted the data, critically revised the first draft of the manuscript, and contributed to the preparation of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.821428/full#supplementary-material

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Evaluation of Additional *Drosophila suzukii* Male-Only Strains Generated Through Remobilization of an FL19 Transgene

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Drosophila suzukii (D. suzukii) (Matsumura, 1931; Diptera: Drosophilidae), also known as spotted wing *Drosophila*, is a worldwide pest of fruits with soft skins such as blueberries and cherries. Originally from Asia, *D. suzukii* is now present in the Americas and Europe and has become a significant economic pest. Growers largely rely on insecticides for the control of *D. suzukii*. Genetic strategies offer a species-specific environmentally friendly way for suppression of *D. suzukii* populations. We previously developed a transgenic strain of *D. suzukii* that produced only males on a diet that did not contain tetracycline. The strain carried a single copy of the FL19 construct on chromosome 3. Repeated releases of an excess of FL19 males led to suppression of *D. suzukii* populations in laboratory cage trials. Females died as a consequence of overexpression of the tetracycline transactivator (tTA) and tTA-activated expression of the *head involution defective* proapoptotic gene. The aim of this study was to generate additional male-only strains that carried two copies of the FL19 transgene through crossing the original line with a *piggyBac* jumpstarter strain. Males that carried either two chromosome 3 or a singleX-linked transgene were identified through stronger expression of the red fluorescent protein marker gene. The brighter fluorescence of the X-linked lines was likely due to dosage compensation of the red fluorescent protein gene. In total, four X-linked lines and eleven lines with two copies on chromosome 3 were obtained, of which five were further examined. All but one of the strains produced only males on a diet without tetracycline. When crossed with wild type virgin females, all of the five two copy autosomal strains examined produced only males. However, the single copy X-linked lines did not show dominant female lethality. Five of the autosomal lines were further evaluated for productivity (egg to adult) and male competition. Based on these results, the most promising lines have been selected for future population suppression experiments with strains from different geographical locations.

Keywords: *Drosophila* suzukii, piggyBac transposon, sterile insect technique, spotted wing drosophila, fsRIDL

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INTRODUCTION

First reported in 2008 in California and Europe, *Drosophila suzukii (D. suzukii)* is now widely found through North America, Europe and some locations in South America (Cini et al., 2014; Asplen et al., 2015; Tait et al., 2021). Unlike most *Drosophila* species that are not economic pests, *D. suzukii* females lay their eggs in ripe fruit before harvest (Hauser, 2011). The species is commonly known as

spotted wing *Drosophila* since adult males have a dark spot that is clearly seen on each wing (Hauser, 2011). Growers largely rely on insecticides for control but use is weather-dependent and resistance to the chemicals is anticipated as seen with Spinosad in California (Gress and Zalom, 2019). *D. suzukii* have a wide range of non-crop host plants, which can serve as a refuge (Lee et al., 2015; Kenis et al., 2016). Thus, reinfestation of crops following insecticide treatment can be relatively rapid (Tait et al., 2021). Additional area-wide control methods are clearly needed.

One promising approach for area-wide control of insects is the release of fertile males carrying dominant female lethal genes (Heinrich and Scott, 2000; Thomas et al., 2000), which is also known as fsRIDL (female-specific release of insects carrying a dominant lethal genetic system) (Alphey, 2014). Wild type virgin females that mate with released fertile fsRIDL males will only produce male offspring. Modeling indicates that repeated releases of an excess of fsRIDL males can lead to suppression of pest populations (Schliekelman and Gould, 2000; Thomas et al., 2000). The fsRIDL strains can be reared in the laboratory or a mass-rearing facility as a conditional system is used for controlling expression of the female-specific lethal gene. Conditional expression is achieved by using the tetracycline transactivator (tTA), a transcription factor that binds very specifically to a sequence from the *Escherichia coli* tet operator (tetO) (Gossen and Bujard, 1992). The binding of tTA to the tetO is inhibited by adding tetracycline to the diet, thus providing a simple off-switch. In the initial system we developed (Heinrich and Scott, 2000), the lethal or effector gene cassette consisted of seven copies of tetO, a core promoter and the coding sequence for the *head involution defective* (*hid*) proapoptotic gene. Widespread expression of *hid* in *D. melanogaster* led to organismal death (Grether et al., 1995). Female-specificity was achieved by using an enhancer-promoter from a yolk protein gene to drive tTA expression (Heinrich and Scott, 2000). Subsequently, fsRIDL strains were simplified to a single component system that consisted of a tTA activated enhancer-promoter driving expression of tTA (Fu et al., 2007; Ant et al., 2012). In this autoregulatory system, very high levels of tTA gene expression led to organismal death likely due to "transcriptional squelching" or inhibition of ubiquitin-mediated proteolysis (Fu et al., 2007). Only females died on a diet without tetracycline as the tTA coding region was interrupted by the sex-specifically spliced first intron from the *Ceratitis capitata transformer* (*tra*) gene (Fu et al., 2007). Similarly, the initial New World screwworm (*Cochliomyia hominivorax*) fsRIDL strains carried single component tTA overexpression transgenes but with the sex-specific intron from the *C. hominivorax tra* gene (Concha et al., 2016). The female-specific tTA overexpression systems were functional in *D. melanogaster*, indicating that the screwworm and *C. capitata tra* introns were correctly spliced in *D. melanogaster* (Fu et al., 2007; Li et al., 2014). We recently developed the FL19 *D. suzukii* fsRIDL strain that had a female-specific tTA overexpression gene and a tTA activated *hid* gene in a single construct (Li et al., 2021).

The effectiveness of fsRIDL strains for population suppression has been demonstrated in cage trials. In the continuous population experiments, conditions were first established for maintaining a population simply by providing sufficient diet. Subsequently, repeated releases of an excess of fertile fsRIDL males led to eradication of the populations (Ant et al., 2012; Leftwich et al., 2014; Harvey-Samuel et al., 2015). With *D. suzukii*, repeated releases of FL19 males (approximately 10–13:1 ratio) led to a sharp reduction in egg production in the first month and by 8 weeks the test cages had stopped laying eggs (Li et al., 2021). Males from a fsRIDL strain of the diamondback moth, *Plutella xylostella*, have been tested in cages (Harvey-Samuel et al., 2015) and in the open field (Shelton et al., 2020). For the latter, the fsRIDL males showed excellent dispersal and persistence (Shelton et al., 2020).

In the field, fsRIDL males will likely encounter females from populations with much greater genetic diversity than found in lab strains (Lewald et al., 2021). To investigate the sensitivity of a female-specific tTA overexpression system to variation in genetic background, we utilized the *D. melanogaster* Genetic Reference Panel (DGRP) that consists of 205 highly inbred lines each with fully sequenced genomes (Mackay et al., 2012). Males from an fsRIDL strain were crossed with virgin females from each DGRP line and the number of male and female offspring counted. The level of female lethality between DGRP lines varied considerably from 11% to 97% with a broad sense heritability of 0.89 (Knudsen et al., 2020). We concluded that genetic background could have a significant impact on the efficacy of the tTA overexpression system. This was one reason why a second effector, *hid*, was included in the FL19 construct. The aim of this study was to develop robust *D. suzukii* fsRIDL strains that either carried two copies of the FL19 transgene or carried the FL19 transgene at a favorable chromosomal location as the tTA expression system is sensitive to position-effects (Heinrich and Scott, 2000; Horn and Wimmer, 2003). This was achieved by remobilizing the original FL19 transgene through crossing with a *piggyBac* jumpstarter strain that expresses *piggyBac* transposase in the germline (Chu et al., 2018). Here we report on the new *D. suzukii* fsRIDL strains obtained by using this approach.

MATERIALS AND METHODS

Fly Rearing, Transposition and Recombination Mapping

D. suzukii were raised on cornmeal-yeast-agar diet at room temperature (approx. 20–22°C) in the open laboratory. The relative humidity in the lab was between 20% and 50% and the lights were on for about 12 h on most days. The original wild-type colony was established from infested fruit collected from a field in North Carolina in 2011 (Burrack et al., 2013) and the initial FL19 strain was previously made by *piggyBac*-mediated germline transformation of this wild type strain (Li et al., 2021). The wild type colony was periodically genetically augmented with flies collected in North Carolina (Elsensohn et al., 2021). The newly refreshed and the original 2011wild type colonies are maintained separately by our lab. To remobilize the FL19 transgene, ten FL19 virgin females were crossed with five males from the H7 *piggyBac* jumpstarter strain (Chu et al., 2018) (**Figure 1**). From the offspring of the cross, ten virgin females were crossed to five wild type males. The male offspring were screened for bright red fluorescence using a M205FA microscope (Leica Microsystems, Buffalo Grove, IL) with

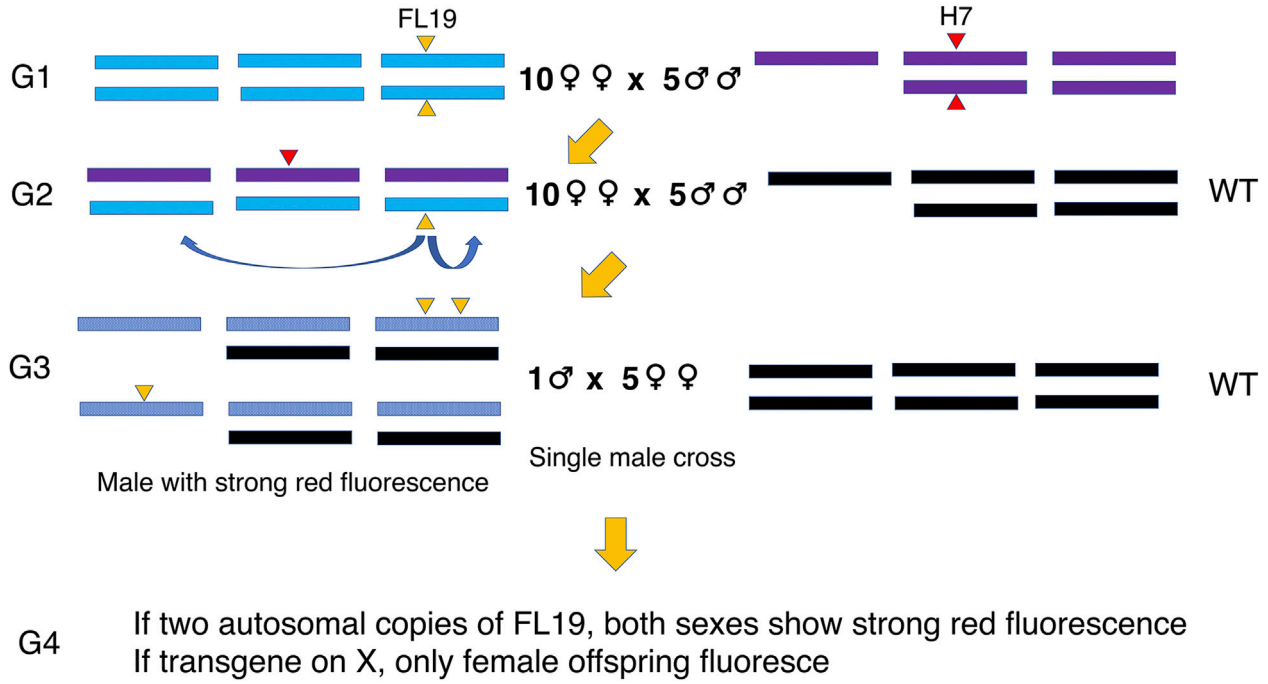

FIGURE 1 | Mating scheme to produce and identify males with a transposed FL19 transgene. FL19 females were crossed with the H7 *piggyBac* transposase jumpstarter males to initiate transposition. Males that could be carrying an X-linked or two autosomal copies of FL19 were identified by fluorescence intensity in G3.

the DsRed filter [ex 545/25, em 595/50 nm]. Individual candidate males were then each crossed with five wild type virgin females. If the FL19 transgene had transposed to the X chromosome, then only the female offspring would show red fluorescence. If both sexes showed bright red fluorescence, this could indicate that the flies carried two autosomal copies of FL19. Homozygous lines for each putative transposition event were established by crossing and selecting for particularly high levels of red fluorescence.

For recombination mapping of X-linked FL19 transgenes, six crosses were set for each combination FL19(X) lines. The double heterozygous virgin female offspring were collected and crossed with wild type males. Recombinant male offspring that showed no red fluorescence was identified and counted. A map distance of the two FL19(X) sites were calculated by; map distance (cM) = 100 x (2 x number of non-fluorescent males)/total males.

Assessment of Female-specific Lethality

To assess the level of female lethality in the homozygous strains, three vials were set on the same day with five pairs on a diet that either contained tetracycline (40 μg/ml) or lacked tetracycline. Parents were transferred three times to new cultures every 3 days to create four cultures. To determine if the strains showed dominant female lethality, five transgenic males were crossed with five wild type virgin females. Parents were transferred to new cultures every 3 days to create four cultures. Two replicates were set each on tetracycline and non-tetracycline foods. The number of male and female offspring from each cross were counted daily until 20 days after setting the cross.

Assessment of Strain Productivity

Cut vials, which were regular fly vials cut at the middle to create 7.2 cm-long tubes and 2.3 cm-deep cups, were used for easy handling of eggs. A tube and a cup were taped together to create a vial for productivity tests. The cup contained about 10 ml of culture medium. Flies that were raised under non-crowded conditions were collected from several vials 3 to 14 days after eclosion and then allowed to lay eggs in the cut vials for about 20 h. The cut vials were then separated, eggs were picked with a needle and transferred to a new vial with about 10 ml of medium. Typically, 50 eggs were transferred to each vial. After 2 days unhatched eggs were counted. Emerging adults were sexed and counted up to 3 weeks after the egg-picking. At the end of emergence of adult flies, the number of pupal cases was counted. The egg survival ratio is the number of hatched eggs divided by the total number of eggs. The larval survival ratio is the number of pupae divided by the number of hatched eggs. The pupal survival ratio is the number of adults divided by the number of pupae. The egg to adult survival ratio is the number of adults divided by the total number of eggs. This ratio was multiplied by two for the transgenic lines on diet without tetracycline.

Male Mating Competitiveness

Ten transgenic males from strains reared without tetracycline in the diet and 10 wild type males were introduced into an 8 oz bottle with diet and left undisrupted for 1 hour. Ten wild type virgin females were added to the bottle which was kept undisrupted at

room temperature (~22°C) for approximately 24 h. All flies were four to 6 days old. Females were transferred individually to fresh vials with diet. The offspring of each female were counted, sexed and examined for fluorescence status (presence/absence) to determine whether the female had mated with an FL19(3 + 3) or wild type male. The presence of both fluorescent and non-fluorescent offspring would indicate remating during the 24 h period when males were with females. Four to eight bottles were set for each line. Mate success ratio of each line was calculated by; success ratio = (number of females with fluorescent sons only + a half number of females with both fluorescent and non-fluorescent sons)/number of fertile females.

Molecular Analysis

The genomic location of the transgenes was determined using inverse PCR with primers for the *piggyBac* left and right ends as previously described (Li et al., 2001 and see **Supplementary Table S1**). If inverse PCR was only successful for one end, confirmation of the transgene location was obtained by PCR using one primer for the *piggyBac* end and one primer based on the flanking genome sequence of the insertion site.

Statistical Analysis

For the female lethality tests (**Table 4**), contingency analysis (fit Y by X) was performed using JMP Pro 15 (SAS Institute). The egg to adult ratio (**Table 5**) was analyzed in SAS (Version 9.4, Cary, NC) on a diet with tetracycline, with the total number of adults divided by the number of eggs as the response variable in PROC LOGISTIC, and both line, tetracycline (+/-), and their interaction as predictor variables. All effects were statistically significant ($p < 0.0001$). For diet without tetracycline, the number of male eggs was estimated as the floor of the number of eggs divided by two. Least-squares means were obtained for each treatment combination, and the specific differences of interest were calculated. The log-odds ratio of the two treatments (each line compared to each wild type for both tetracycline + and tetracycline -) were obtained. To control for multiple tests, a Bonferroni correction was used where the adjusted p-value to determine statistical significance was set at 0.05/30 = 0.0016 for a diet with tetracycline and 0.05/15 = 0.0033 for a diet without tetracycline (only males produced from transgenic lines). The male competitiveness data (**Table 6**) were also analyzed in SAS, Version 9.4 (Cary, NC). The mating competitiveness index (MCI) was recalculated as the ceiling of the number mated with transgenic plus half the number that remated divided by the total number mated. This allowed the count to remain an integer. A z-test for one proportion was run for each line comparing the MCI to the null hypothesized proportion of 0.5.

RESULTS

Transposition of the FL19 Transgene to New Chromosomal Locations

The original FL19 male-only strain was made by *piggyBac* transposase-mediated germline transformation (Li et al., 2021). The transgene was located on chromosome 3 between the *DsShal* and *DsCG9231* genes. The *piggyBac* H7 jumpstarter strain efficiently mediates remobilization of *piggyBac* transgenes (Chu et al., 2018). To remobilize the FL19 transgene, FL19 virgin females were crossed with H7 males and virgin female offspring collected (**Figure 1**). As X-linked transgenes are generally dosage compensated in male *Drosophila melanogaster* (Spradling and Rubin, 1983; Fitzsimons et al., 1999), we reasoned that G3 males carrying a single X-linked FL19 transgene would show an increased expression of the red fluorescent protein marker gene (**Figure 1**). Males that carried FL19 at the original location and at a second autosomal location would also show brighter red fluorescence. Such transpositions tend to be predominately local (Daniels and Chovnick, 1993), so the expectation was that males would carry FL19 at the original location and at a closely linked site on chromosome 3. X-linked transposition events were identified through crossing putative males with wild type virgin females (**Figure 1**). From approximately 25,000 G3 males, we derived four X-linked lines and eleven lines with two copies of FL19 on chromosome 3 (**Table 1**). Of the third chromosome lines, one was homozygous lethal and two lines were homozygous sterile (**Table 1**). Of the remaining eight homozygous viable lines, five (8, 36, 40, 70 and 75) that were vigorous on diet with tetracycline were selected for further study.

TABLE 1 | X-linked and third chromosome FL19 lines.

Chromosome	Line	Homozygous condition
X	7	viable
X	46	viable
X	77	viable
X	79	viable
3	F8	sterile
3	6	sterile
3	8	viable
3	17	viable
3	18	dead
3	36	viable
3	40	viable
3	70	viable
3	75	viable
3	78	viable
3	83	viable

The chromosomal locations of the X-linked and most of the chromosome 3 lines were determined by inverse PCR and blast searches of the assembled *D. suzukii* genomes (Chiu et al., 2013; Paris et al., 2020) (**Table 2**). Some locations could not be determined as the transgene appeared to be located within a repetitive sequence. PCR analysis confirmed that all the chromosome 3 lines carried two copies of FL19, with one copy at the original location near the *DsCG9231* gene (**Figure 2**). The other copies of FL19 were found to have inserted no more than 68 kb from the original location (**Table 5**; **Figure 2**). In line 36, the additional FL19 transgene is less than 3 kb from the original and is also within the intergenic region between the *DsCG9231* and *DsSha1* genes (**Figure 2**). In line 70, the additional FL19 transgene is also in an intergenic region, between the *Dswnd* and *DsRnf146* genes (**Table 2**; **Figure 2**). In the other lines, 8 and 75,

TABLE 2 | FL19 insertion sites in transposition lines.

Line	Chromosome	Insertion site sequence (TTAA in bold)	Nearest gene	Relationship to original FL19 location
7	X	TCGATATCAGGTGGTGCACT**TTAA**GGAGTTGGAGCATAGCATAT	*DsCG8661* (contig 7[a])	NA
46	X	GCTCCGCCGTCGTTTGTATT**TTAA** TTTAGCCTCTTCAAATTGCT	*DsCG32655* (>20 kb) (contig 11)	NA
77	X	CGCCAAAACGCAAGAAACCT**TTAA**AAGAGTAATCCAGATAATGG	*DsCp110* (contig 15)	NA
79	X	ND (repetitive)	ND	NA
8	3	TAAATAATTTCGAAACCACT**TTAA**AAAGAACTTTGTAGTTTAGT	*DsRnf146* (intron)	67.8 kb 3′
36	3	TTGTAAATTAAAATAAAGGC**TTAA**CTAAAAAAAGTACCAAGAAC	*DsCG9231*	2.6 kb 5′
40	3	ND	ND	ND
70	3	GAGGATCATGTTGATGCCCA**TTAA**ACCGGCCAAGCTCAGAAGCA	*Dswnd* and *DsRnf146*	60.8 kb 3′
75	3	CGTGTTTACCGGTTCGTGC**TTAA**ACTTGAATTCCCGAAGAGAT	*DsCG14100* (coding)	7.5 kb 3′

[a]*Contigs of the Paris et al. (2020) genome assembly.*

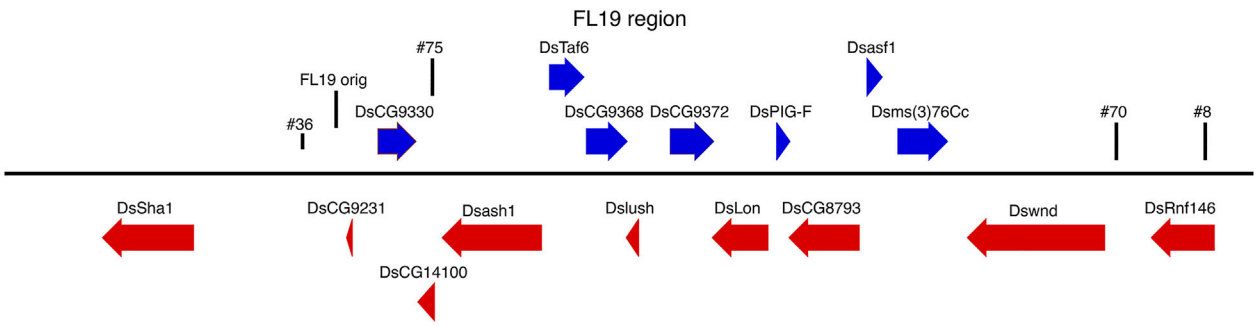

FIGURE 2 | Relative location of FL19 transgenes on chromosome 3. The location of transgenes is indicated by vertical lines. The length and direction of arrows indicates the sizes and direction of transcription of transcription of genes as annotated (Paris et al., 2020).

TABLE 3 | Recombination mapping of X-linked FL19 transgenes.

Cross	Number fluorescent F_1 males	Number wild type F_1 males	Map distance (cM)
79♀ x 46♂	114	33	45
46♀ x 7♂	99	18	31
79♀ x 7♂	151	9	11
77♀ x 79♂	136	0	0
7♀ x 77♂	114	5	8

the transgene is located within genes. In line 8, the transgene is within an intron of the *DsRnf146* gene. In the line 75 the transgene is within an exon of the *DsCG14100* gene and would likely disrupt gene function.

In the X-linked lines, three of the four FL19 transgenes appear to be found at widely separated locations as their flanking sequences each align to a different contig of the genome assembly (Paris et al., 2020) (**Table 2**). The location of the fourth transgene (#79) could not be determined by inverse PCR. In line 46 the transgene is in a gene-poor region of the X chromosome with the nearest genes more than 20 kb distant. We next used recombination mapping to determine the genetic distance between each of the transgenes. The transgene in line 46 was found to be 45 cM from the transgene in line 79 (**Table 3**). The transgenes in lines 77 and 79 appear to be very closely linked locations as we did not recover any recombinants (**Table 3**). Lastly, in line 7 the transgene mapped to a region between the 46 and 79 transgenes (**Table 3**).

Tetracycline-Repressible Female Lethality

All the lines can be readily maintained on diet supplemented with tetracycline. When raised on diet that lacked tetracycline all lines produced 99–100% males except for the X-linked line 46, which gave 73.5% males (**Table 1**). In a future field release, flies would be raised on diet without tetracycline and the released fertile males would mate with wild type females. Ideally, all the female offspring would die. Therefore, we next collected males from the lines raised on diet without tetracycline and crossed to wild type virgin females. For the chromosome 3 lines that carry two copies of the FL19 transgene, all lines showed dominant female

TABLE 4 | Tetracycline-repressible female-specific lethality of FL19 transposition lines.

Strain (chromosome)	Tetracycline	Homozygous Number Males[a]	Homozygous Number Females	Homoyzgous % Male	Hemizygous Number Males	Hemizygous Number Females	Hemizygous % Males
7 (X)	–	239	0[c]	100	239	81[c]	74.7
	+	253	221	53.3	294	286	50.7
46 (X)	–	119	43[c]	73.5	186	229[d]	44.8
	+	188	234	44.5	192	206	48.2
77 (X)	–	268	0[c]	100	236	52[c]	82
	+	283	243	53.8	264	275	49
79 (X)	–	261	1[c]	99.6	226	274[d]	54.8
	+	188	242	43.7	274	289	48.7
7 (X) + FL19	–	66	0[c]	100	115	0[c]	100
	+	54	42	56.2	146	166	46.8
8 (3)	–	60	0[c]	100	179	0[c]	100
	+	46	55	45.5	263	258	50.5
36 (3)	–	64	0[c]	100	245	0[c]	100
	+	61	32	65.6	198	248	44
40 (3)	–	34	0[b]	100	51	0[c]	100
	+	74	34	68.5	139	161	46.3
70 (3)	–	40	0[b]	100	190	0[c]	100
	+	89	29	75.4	258	225	53.4
75 (3)	–	20	0[b]	100	176	0[c]	100
	+	49	21	70	257	290	53.8

[a]*Total count of offspring from three independent vials of flies except for the homozygous chromosome 3 and X + FL19 lines where the data is from the productivity experiment shown in* ***Table 1****.*

[b]*The number of females obtained was significantly lower than expected (Pearson's Chi-squared test,* $p < 0.001$*).*

[c]*The number of females obtained was significantly lower than expected (Pearson's Chi-squared test,* $p < 0.0001$*).*

[d]*The number of females obtained was not significantly lower than expected.*

lethality (**Table 4**). However, none of the X-linked single copy lines showed dominant female lethality. Two lines, 7 and 77, produced significantly more male than female offspring (Pearson's Chi-squared test, $p < 0.0001$). On a diet with tetracycline, approximately an equal number of males and females were produced from the crosses of transgenic males with wild type females.

General Fitness and Male Sexual Competitiveness of Transgenic Sexing Strains

One of the fitness measurements that is important in a mass rearing facility is the percentage of eggs that produce adults (Concha et al., 2016; Concha et al., 2020). We firstly measured the proportion of eggs that produced adults in our two wild type North Carolina colonies. One colony has been periodically refreshed with flies collected in North Carolina and the other has been continuously maintained in the laboratory since 2011. The 2011 wild type strain showed significantly reduced adult production compared to the newly refreshed strain on diet with or without tetracycline ($p < 0.0001$) (**Table 5**). Similarly, on diet with tetracycline, the adult production for all of the transgenic lines was significantly less than the newly refreshed wild type strain (**Table 5**). However, since the original FL19 line was by injecting embryos from the 2011 wild type strain, we also compared each of the transgenic lines to that strain. Compared to the 2011 wild type strain, adult production on a diet with tetracycline and male production on a diet without tetracycline was not significantly different in five of the seven lines (FL19, 8, 36, 40, 7(X) + FL19). Two of the transgenic lines, 70 and 75, did show significantly reduced adult production on both diets compared to the 2011 wild type strain (**Table 5**). Several of the transgenic lines (e.g., 8, 40, 70) showed a wider variation between replicates for pupal and adult survival on a diet without tetracycline than on a diet with tetracycline (see standard deviation (SD) values in **Table 5**). This variability in male survival suggests there may be some leaky expression of the lethal genes, particularly in some lines.

The sexual competitiveness of the males from the lines was assessed by presenting virgin wild type females with equal numbers of transgenic and wild type males as done previously with the *C. hominivorax* male-only strains (Concha et al., 2016). As previously, the MCI was calculated where an index of 0.5 indicates the transgenic and wild type males are equally competitive. Males from the original FL19 strain and from most of the new male-only lines were significantly out-competed by the wild type males (**Table 6**). However, males from line 36 and the combined 7 (X-linked) with the original FL19 strain competed effectively with the wild type males, with MCI values not significantly different than the expectation of 0.5 if fully competitive (**Table 6**).

DISCUSSION

In this study, the FL19 transgene reported recently (Li et al., 2021) was remobilized to new locations on the third and X chromosomes. As expected, the third chromosome jumps were relatively short with the most distant transgene less than 70 kb

TABLE 5 | Productivity of FL19 transposition lines.

Strain (chromosome)	Tetra-cycline	Number eggs[a]	Number unhatched eggs	Number pupae	Number males	Number females	Number total adults	Egg survival ratio Mean (SD)	Larval survival ratio Mean (SD)	Pupal survival ratio Mean (SD)	Egg to adult ratio[b] Mean (SD)
Wild type	–	150	10	133	65	59	124	0.93 (0.046)	0.95 (0.111)	0.93 (0.033)	0.83 (0.11)
	+	183	15	165	67	89	156	0.92 (0.023)	0.97 (0.1)	0.95 (0.051)	0.85 (0.103)
Wild type (est.	–	230	30	157	69	70	139	0.87 (0.064)	0.79 (0.1)	0.89 (0.076)	0.62** (0.121)
2011)	+	200	20	117	43	62	105	0.84 (0.054)	0.70 (0.082)	0.90 0.11)	0.53** (0.079)
7 (X) + FL19 (3)	–	200	16	70	66	0	66	0.92 (0.016)	0.38 (0.074)	0.94 (0.011)	0.66^{NS} (0.12)
	+	200	14	112	54	42	96	0.93 (0.035)	0.6 (0.178)	0.87 (0.091)	0.48^{NS} (0.099)
FL19 (3)	–	600	63	186	157	0	157	0.89 (0.041)	0.35 (0.1)	0.86 (0.11)	0.52^{NS} (0.145)
	+	600	70	350	161	150	311	0.88 (0.05)	0.66 (0.106)	0.89 (0.073)	0.52^{NS} (0.08)
8 (3)	–	282	18	83	60	0	60	0.94 (0.049)	0.32 (0.13)	0.66 (0.3)	0.44^{NS} (0.32)
	+	200	16	112	46	55	101	0.92 (0.051)	0.61 (0.13)	0.9 (0.06)	0.51^{NS} (0.117)
36 (3)	–	281	70	64	54	0	54	0.75 (0.071)	0.3 (0.13)	0.87 (0.14)	0.37^{NS} (0.135)
	+	146	36	61	27	32	59	0.75 (0.05)	0.55 (0.06)	0.97 (0.03)	0.40^{NS} (0.053)
40 (3)	–	142	50	34	22	0	22	0.65 (0.08)	0.37 (0.09)	0.60 (0.42)	0.3^{NS} (0.27)
	+	166	53	74	36	34	70	0.68 (0.023)	0.66 (0.113)	0.96 (0.057)	0.42^{NS} (0.051)
70 (3)	–	200	48	40	23	0	23	0.76 (0.059)	0.26 (0.088)	0.53 (0.33)	0.23* (0.21)
	+	236	69	89	56	29	85	0.71 (0.069)	0.53 (0.083)	0.96 (0.057)	0.36* (0.071)
75 (3)	–	217	90	20	10	0	10	0.59 (0.102)	0.17 (0.117)	0.43 (0.159)	0.1* (0.092)
	+	200	79	48	25	21	46	0.6 (0.066)	0.4 (0.047)	0.94 (0.04)	0.23* (0.038)

[a]*Total count of offspring from at least three replicates.*

[b]*On diet without tetracycline this is the number of males divided by the number of eggs times two. On diet with tetracycline this is the total number of adults divided by the number of eggs. NS, indicates not significantly different compared to wild type (est. 2011). * indicates significantly reduced adult production compared to wild type (est. 2011) (*$p < 0.0016$ *for + tetracycline and* $p < 0.0033$ *on no tetracycline, see methods for details). ** indicates significantly reduced adult production compared to wild type (newly established).*

TABLE 6 | Male sexual competitiveness.

Line (chromosome)	Number replicates	Number mated with transgenic	Number mated with wild type	Number mated with both males (remating)	Total mated	MCI[a] (SE)	*p*-value
FL19 (3)	7	16	48	3	67	0.27 (0.06)	0.0002
7 (X) + FL19 (3)	4	14	23	1	38	0.39 (0.08)	0.1944
8 (3)	6	16	40	0	56	0.29 (0.07)	0.0013
36 (3)	5	25	18	1	44	0.59 (0.08)	0.2278
40 (3)	8	15	46	9	70	0.29 (0.06)	0.0003
70 (3)	7	14	46	3	63	0.25 (0.06)	<0.0001
75 (3)	4	7	26	1	34	0.24 (0.09)	0.0020

[a]*Mating competitiveness index or MCI, is the number mated with transgenic plus half the number that remated divided by the total number mated.*

from the original FL19 location. All chromosome 3 lines had two transgenes, with one at the original location. This could occur by transposition to the non-donor sister chromatid during meiosis after DNA replication as seen with *P* element transposition (Daniels and Chovnick, 1993). Alternatively, transposition could occur to the donor sister chromatid followed by repair using the non-donor sister chromatid as template. All chromosome 3 lines showed 100% dominant female lethality when transgenic males were crossed with wild type females on diet without tetracycline. The most promising of the five lines examined are 8, 36 and 40 as the level of production of adults from eggs was comparable to the original wild type strain. Of these, line 36 males were sexually competitive with wild type males. Males from all other chromosome 3 lines, including the original FL19 strain, were significantly less competitive than wild type males. It is not obvious why line 36 males would be more competitive than males from the original FL19 line. Perhaps by chance, changes in the genetic background produced a strain with improved male competitiveness. If so, it may be beneficial to backcross transgenic males with females from the recently refreshed wild type strain, which showed significantly higher productivity compared to the wild type strain that has been in the lab for about 10 years. While none of the X-linked lines showed dominant female lethality, two lines, 7 and 77, produced mostly males. Further, 100% of homozygous females died when reared on diet without tetracycline. While not desirable for fsRIDL, these lines could be considered for a sterile release program with the males sterilized by exposure to radiation (Sassu et al., 2019). The level of dominant female lethality could be increased by making a recombinant strain that carries two transgenes, one each from the 7 and 77 lines. However, this could be challenging without balancer chromosomes. If female lethality is fully dominant, matings with wild type females would produce only males that would not carry any transgenes. This could be desirable if there is concern about transgene persistence in the field (Evans et al., 2019). Since line 7 showed a high level of female lethality, we combined it with the original FL19 line and bred to homozygosity for both transgenes. This strain, 7 (X) + FL19, appears to be quite promising as productivity was comparable to the long established wild type strain and males were sexually competitive with wild type males. A disadvantage of this strain for an fsRIDL program is that the male offspring from matings with wild type females would only inherit the chromosome 3 transgene.

The frequency of remobilization seen in this study was much lower than reported previously with the H7 *piggyBac* jumpstarter strain (Chu et al., 2018). This was most likely because our screen would have missed simple cut and paste transposition events to autosomal locations as most would not have produced males with significantly increased expression of the red fluorescent protein gene. In contrast, Chu et al., 2018 used donor strains that carried a fluorescent protein gene that was sensitive to position-effects, likely nearby transcription enhancers. Consequently, transposition events could be detected by changes in the expression pattern of the fluorescent protein gene.

One aim of this study was to produce strains with two copies of the FL19 transgene that could be tested against strains with different genetic backgrounds. For example, by crossing transgenic males with virgin female wild type flies from Western and Eastern US populations which are genetically quite distinct (Lewald et al., 2021). Several strains made in this study (8, 36, 40, 7(X)+FL19) would be worth evaluating since they show high female lethality and productivity comparable to the older wild type strain. For fsRIDL, modeling has shown that it would be advantageous if each autosome (i.e. chromosomes 2, 3 and 4) carried a copy of the dominant female lethal transgene (Schliekelman and Gould, 2000; Thomas et al., 2000). This could be accomplished by targeting FL19 to specific chromosomal locations using CRISPR/Cas9. For this approach the FL19 gene construct and fluorescent protein gene would be flanked with homologous sequences from the region targeted rather than the 5′ and 3’ ends of the *piggyBac* transposon as used in this study. Cas9-mediated cleavage of genomic DNA followed by homology-dependent repair using the injected plasmid DNA as template should lead to integration of the FL19 construct at the targeted location (Wang et al., 2015; Davis et al., 2018). These experiments would be facilitated by using the lines that express Cas9 in the germline that we described recently (Kandul et al., 2021).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AY design research, performed the crosses, collected and analyzed the data. AmY carried out the molecular analyses of the strains and analyzed the data. MS designed research, analyzed data, performed some of the statistical analyses, wrote the first draft and obtained funding for this project. All authors read, edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.829620/full#supplementary-material

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REVIEW
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The Challenges in Developing Efficient and Robust Synthetic Homing Endonuclease Gene Drives

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Making discrete and precise genetic changes to wild populations has been proposed as a means of addressing some of the world's most pressing ecological and public health challenges caused by insect pests. Technologies that would allow this, such as synthetic gene drives, have been under development for many decades. Recently, a new generation of programmable nucleases has dramatically accelerated technological development. CRISPR-Cas9 has improved the efficiency of genetic engineering and has been used as the principal effector nuclease in different gene drive inheritance biasing mechanisms. Of these nuclease-based gene drives, homing endonuclease gene drives have been the subject of the bulk of research efforts (particularly in insects), with many different iterations having been developed upon similar core designs. We chart the history of homing gene drive development, highlighting the emergence of challenges such as unintended repair outcomes, "leaky" expression, and parental deposition. We conclude by discussing the progress made in developing strategies to increase the efficiency of homing endonuclease gene drives and mitigate or prevent unintended outcomes.

Keywords: gene drive, gene editing (CRISPR-Cas9), DNA repair, germline, transgene expression, deposition, multiplexing

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1 INTRODUCTION

Gene drive is the ability of a genetic element to bias its own inheritance. This allows gene drive elements to spread a genetic change through a population even while having a fitness disadvantage ("selfish-DNA"). Genetic engineering at scale through engineered/synthetic gene drives may allow many currently intractable public health challenges caused by pest species to be addressed. In particular, insect pests such as mosquitoes have life-history traits that may make them amenable to gene drive interventions (e.g., sexual reproduction and short generation times). The feasibility of using gene drives to fix a particular trait (population replacement) or suppress wild populations are both being investigated for addressing the harm caused by insect pests, in some cases with the same ultimate goal (e.g., eradication of malaria).

There are many examples of gene drives occurring in nature, acting through many different mechanisms (Burt and Trivers, 2006). Some types of gene drive rely on the action of sequence-specific DNA nucleases (enzymes that create DNA breaks). These have recently received a lot of attention by researchers following the discovery and characterisation of Clustered Regularly

Interspaced Short Palindromic Repeats (CRISPR) systems (Jinek et al., 2012). The programmable CRISPR nucleases, of which CRISPR associated protein 9 (Cas9) is the most widely used, have provided researchers with powerful new tools to both facilitate genetic engineering and as constituent parts of gene drive mechanisms. Nonetheless, many important fundamental insights into building synthetic gene drive systems were gained before the use of CRISPR nucleases.

Double-stranded DNA breaks are a common occurrence in cells, and a range of mechanisms exist to resolve them. Under specific conditions, cells can use a homologous DNA template to prevent the loss of genetic information. This can be from an identical sister chromatid that is present during the S and G2 phases of the cell cycle, or the near identical homologous chromosome. Generally, in diploid organisms, each chromosome in a homologous pair is contributed by a different parent and contains the same content with minor sequence variation (sex chromosomes often are an exception). Therefore, interchromosomal repair within a homologous pair will result in loss of heterozygosity, but under most circumstances results in the genomic region retaining its function after repair.

Homing endonuclease gene drives (HEGs) can induce their own switch from a heterozygote to a homozygote state by creating a DNA break in the "recipient" homologous chromosome corresponding to the locus of the HEG genetic material on the "donor" homologous chromosome (**Figure 1A**). In effect, the coding sequence for the HEG may then be identified as missing from the cut chromosome and the HEG and linked sequences are copied over during repair of the DNA break (**Figure 1B**). If the transformed cell is part of the organism's germline lineage, the gene drive element will be propagated to the next generation with a higher frequency than would be expected from normal Mendelian inheritance. This copying or "homing" process can repeat itself in subsequent generations and allows the HEG element to increase in frequency in a population, along with any associated genetic modifications that affect the desired change in the population.

In general, the HEG drives we describe here are designed and optimised for the homing inheritance bias mechanism. However, there are a number of ways through which nuclease-based drives have been described to bias their inheritance with seemingly subtle changes underlying the difference in mechanism. For almost all HEG studies, there is limited evidence on the actual underlying mechanism(s) giving rise to any observed inheritance bias and recent evidence suggests the mechanisms may be more heterogeneous than previously understood. An important hallmark of the homing process is the copying of the drive element onto the recipient chromosome. Many other nuclease drive mechanisms instead operate through decreasing the inheritance of the nondrive recipient chromosome. We will use the term inheritance bias or estimated homing when the specific experimental set-up was not strictly able to distinguish between inheritance bias through copying (homing) or exclusion of the chromosome not carrying the drive allele.

Synthetic HEGs have, in almost all cases, been inserted into and targeted the sequence of an endogenous gene or targeted a separately inserted synthetic target gene (e.g., GFP). A principal reason for this is DNA sequence constraints. Many simultaneous DNA breaks in the genome may result in DNA damage-induced cell stress (Aguirre et al., 2016) and chromosomal rearrangement (Kosicki et al., 2018). As such, synthetic HEGs are designed to only cut their specific target site and those targets are chosen to be unique within the genome. In addition, for HDR to occur, the region surrounding the DNA break must be (relatively) uniquely matched with the homologous chromosome, as homologous loci elsewhere in the genome may compete as evidenced by homing from non-paired sites (Chan et al., 2011; Lin and Potter, 2016). Lastly, for the drive to affect a significant proportion of a population, its target must also be present in most individuals of the target population. These sequence constraints are generally only found in the (coding sequence) of highly conserved genes.

Beyond the sequence constraint, there are additional benefits that may come from placing HEGs in an endogenous gene. The "effector" function of synthetic HEGs (e.g., female recessive sterility) may be most readily achieved by disrupting a specific endogenous gene directly with the drive element (Burt, 2003). In research contexts, the target is often a gene that provides a phenotypic readout when disrupted. In addition, the chromatin environment associated with an endogenous (expressed) gene may be more permissive to the expression of the inserted transgenes (O'Brien et al., 2018; Brady et al., 2020; Dhiman et al., 2020), and an endogenous gene's promoter may even be directly used to express the drive genes (Nash et al., 2019; Weitzel et al., 2021). The target gene's chromatin context may also influence Cas9 cutting efficiency and DNA repair (Verkuijl and Rots, 2019). Lastly, targeting highly conserved essential genes is one of the most important tools for addressing unintended repair outcomes which will be discussed more later.

Generally, the ability of a synthetic HEG to spread will depend on whether its efficiency at biasing its own inheritance can overcome its associated fitness costs. These costs depend on a number of factors: the particular application will matter, as population modification with a "neutral" modification such as insecticide susceptibility or pathogen resistance will likely impose a far lower fitness cost than a modification designed to suppress the target population (cause a population decline). The actions of the drive machinery itself will also apply some fitness cost, and characteristics of the target species and population, such as size, density, gene flow, and density dependence will all factor into the drive requirements. More complex HEG designs required for "self-limiting" drives (Noble et al., 2019) may also place higher requirements on the drive efficiency. Moreover, the HEG efficiency will also influence the required release frequencies, and the logistical costs and feasibility associated with the use of that particular system. As such, understanding and improving gene drive inheritance biasing efficiency and fidelity may allow for application in currently refractory species, and possibly decrease the cost of already feasible interventions.

To our knowledge, synthetic HEGs have, with varying inheritance biasing efficiencies (in some cases none), been reported in 9 species: *Saccharomyces cerevisiae*, *Candida albicans*, *Arabidopsis thaliana*, *Drosophila melanogaster*,

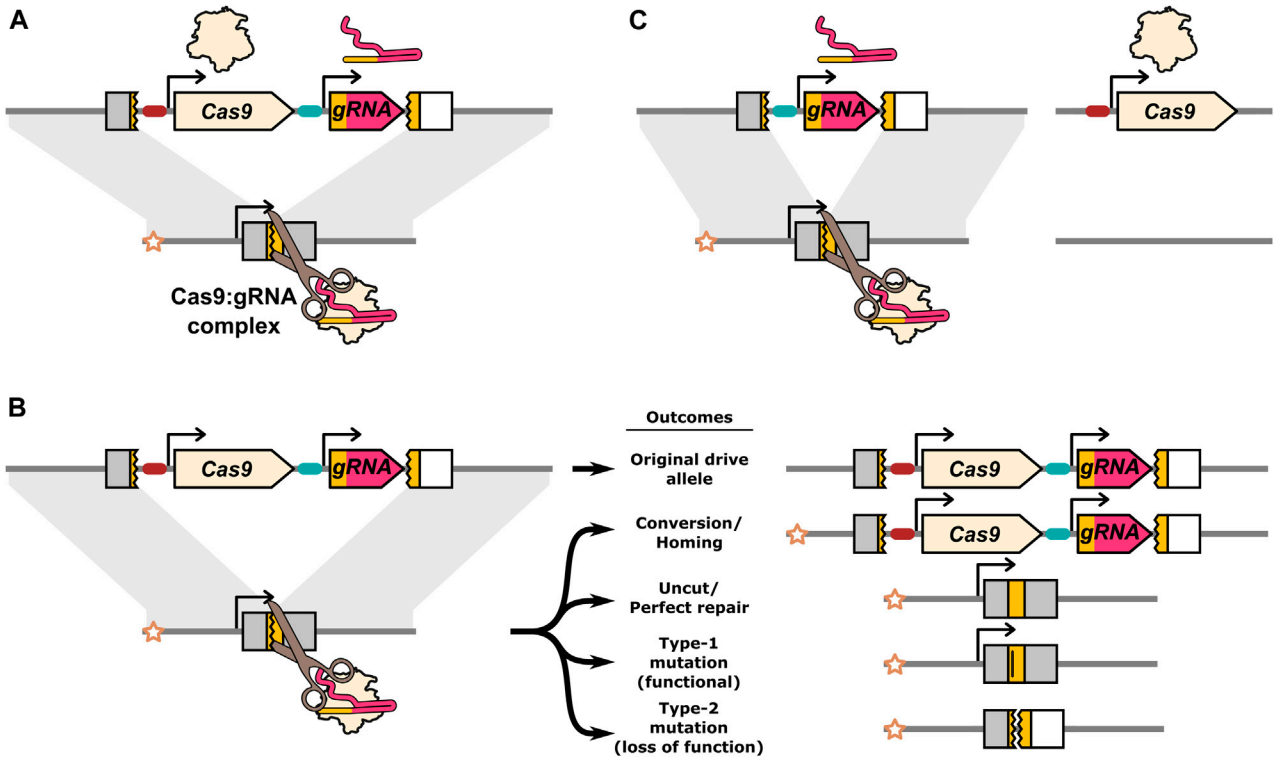

FIGURE 1 | Illustration of the Cas9:*gRNA* homing endonuclease gene drive inheritance biasing mechanism and potential DNA repair outcomes. **(A)** The drive expresses Cas9 and a gRNA which together form a complex that find and cleave the target allele. **(B)** DNA breaks can be resolved by a range of different repair outcomes. Conversion occurs when HDR uses the homologous chromosome carrying the drive allele as a repair template. The star indicates the "recipient" chromosome, and allows the original drive allele to be distinguished from a drive allele produced by homing. Alleles that are cut and repaired perfectly, as well as uncut alleles, remain unconverted. In addition to conversion, DNA repair can also result in mutations in the target gene. If the specific mutations do not disrupt the function of the target gene they are classified as type-1. If the mutations do prevent normal function of the target gene they are classified as type-2. **(C)** A component essential to the inheritance biasing process such as the gRNA or Cas9 gene can be located on a separate element producing a "split-drive" configuration. The gRNA target sequence in the endogenous target gene is indicated by a yellow colouring. The target gene is shaded grey, unless disrupted by the drive or type-2 mutations at which point it is shaded white to indicated its loss of function. Type-1 mutations lose the gRNA target sequence (indicated by a vertical bar in the yellow target site), but remain functional.

Plutella xylostella, *Anopheles gambiae*, *Anopheles stephensi*, *Aedes aegypti*, and *Mus musculus*. The research field has learnt much about the factors influencing HEG outcomes and explored different strategies for optimisation. However, much remains unknown, such as what specifically constitutes an efficient HEG, and what underlies the different outcomes observed with different drive designs. We will first present an overview of the field and the milestones achieved in the development of synthetic HEGs so far. Then we will discuss in detail specific technical challenges in developing efficient HEGs and end with potential solutions.

2 MILESTONES IN THE DEVELOPMENT OF SYNTHETIC HEGS

2.1 Transferring a Natural Homing Endonuclease Gene Drive

Natural HEGs were first identified in unicellular eukaryotes, fungi, and plants (Burt and Trivers, 2006) and it was initially unknown if HEG based inheritance biasing mechanisms would function in animals. *S. cerevisiae* has a natural gene drive that relies on the I-*Sce*I meganuclease, which in its endogenous context cuts the large rRNA sub-unit of the biparentally inherited mitochondria (Monteilhet et al., 1990). Chen et al. integrated the I-*Sce*I nuclease into a synthetic docking site in *D. melanogaster* and separately inserted a fluorescent protein into the docking site to function as a recipient chromosome (Chan et al., 2011). They for the first time demonstrated that a synthetic HEG could bias its own inheritance in animals. In that, and a follow-up study, they performed extensive tests of the I-*Sce*I drive with different regulatory sequences upstream (promoter and 5′UTR) and downstream (3′UTR) of the I-*Sce*I nuclease coding sequence (Chan et al., 2011, 2013a). The best performing drive used the promoter of the Rcd-1r gene and the *β*-Tub56D 3′UTR aiming for spermatogenesis-specific expression. This drive converted 23% of the target alleles located on the recipient chromosome, achieving an overall inheritance of ≈62% (≈50% from donor chromosome + ≈12% converted recipients). The majority (63%) of target alleles on the

recipient chromosome appeared to remain unmodified, likely uncut.

While a substantial number of promoter/3′UTR combinations achieved higher cut rates than Rcd-1r/β-Tub56D, they resulted in lower or even no HEG conversion. Instead of copying the HEG element through HDR, alternative DNA repair pathways created mutations at the site of DNA cleavage. These results indicated that simply creating a DNA break was not enough for efficient homing, the timing of nuclease expression was seemingly essential for efficient conversion. In addition to potentially competing with the homing process, these mutations create cut-resistant alleles that are inherited by the next generation. Mathematical modelling and cage trials have indicated that these cut-resistant alleles can prevent a drive from reaching fixation, or even spreading effectively in real world applications (Marshall et al., 2017; Noble et al., 2017; Champer et al., 2018; Pham et al., 2019; Champer S. E. et al., 2021; Fuchs et al., 2021).

Work by Windbichler et al. demonstrated that the I-*Sce*I HEG could also function in the disease-relevant *A. gambiae* mosquito (Windbichler et al., 2011). Expressed from a male specific promoter, the HEG reached inheritance rates of 86%. Moreover, they showed using small scale cage experiments the first evidence that a synthetic HEG could spread within a receptive population. Despite similar, and many additional promoter/3′UTR combinations having been tested in *D. melanogaster*, the inheritance bias achieved with the *A. gambiae* drive was higher. This was the first suggestion that some organisms are more receptive to HEG based inheritance biasing than others. Yet, even in *A. gambiae* the inheritance bias was likely too low for most applications. Moreover, these HEGs would not function outside of a specifically modified lab strain: both *D. melanogaster* and *A. gambiae* do not naturally contain the I-*Sce*I target sequence and in each case, a synthetic target allele had to be created for the drives to function.

2.2 The Cost of Re-Targeting

To address the targeting limitations, Chan et al. used site-directed mutagenesis of the I-*Onu*I meganuclease to change its recognition sequence to allow it to target a closely related sequence naturally found in *Anopheles* mosquitoes (Chan et al., 2013b). They placed this *Anopheles* target in a GFP reporter and tested the inheritance bias in *D. melanogaster* males using the Rcd-1r promoter and β-Tub56D 3′UTR. While two I-*Onu*I variants biased their inheritance to the same degree as the I-*Sce*I drive (Chan et al., 2011), they only did this with far higher overall cut rates (therefore generating more mutations). Moreover, there were indications that a mutation introduced into the I-*Onu*I catalytic site to achieve these higher cut rates was causing reduced fertility, possibly due to sequence promiscuity. Furthermore, the *Anopheles* gene that the modified I-*Onu*I could target happened to closely match the natural I-*Onu*I targeting sequence and would not in itself confer disease resistance or allow population suppression. Clearly, the creation of synthetic HEGs would greatly benefit from more programmable, yet specific, nucleases.

The first and second generation of programmable nucleases came in the form of zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The only reported use of ZFNs and TALENs in a HEG system was by Simoni et al. (Simoni et al., 2014) with the Rcd-1r promoter and β-Tub56D 3′UTR in *D. melanogaster*. In males, ZFN and TALEN-based HEGs achieved homing rates of 34% and 49%, respectively. These homing rates were higher than with I-*Sce*I, but with equivalent or worse cut-to-homing ratios. While the TALEN HEGs had overall higher homing rates and better cut-to-homing efficiency than the ZFN HEGs, only the ZFN HEGs were able to spread significantly within small cage trials. It became apparent that the programmability of these nucleases came at a cost: repetitive genetic sequences. ZFNs and particularly TALENs are composed of large repeating DNA binding "units" in which only a few amino acids are changed to specify the target sequence. This resulted in repetitive drive constructs, which in turn were found to make the drive unstable, losing function at a high rate due to internal recombination and/or partial homing. Only 40% of the TALEN and 75% of the ZFN inheriting progeny resulting from (partial) homing in the first generation could themselves home in the next generation.

Together, the above work demonstrated that the HEG mechanism could indeed work in animals and spread in small cage populations. However, the difficulty in programming meganucleases, and the shortcomings of ZFNs and TALENs meant that with the available tools it would be a momentous task to create an effective HEG drive system that would spread in non-ideal conditions. That is, until the discovery of the CRISPR nucleases.

2.3 Programmable RNA Guided Gene Drives

Starting in 2012, a new generation of molecular tools greatly accelerated our ability to perform gene editing. Central to this revolution has been the discovery of new easily programmable nucleases, the best-known version being Cas9 from the type-II CRISPR system of *Streptococcus pyogenes* (Jinek et al., 2012). Cas9 can, with few limitations, be targeted to almost any DNA sequence by straightforward RNA to DNA base pairing through a short "guide" RNA (gRNA). If a sufficient match is found (not necessarily perfect), Cas9 will then create a double-stranded break. In a very short time-frame CRISPR based synthetic HEGs were reported in *S. cerevisiae* (DiCarlo et al., 2015), *D. melanogaster* (Gantz and Bier, 2015), *A. stephensi* (Gantz et al., 2015), and *A. gambiae* (Hammond et al., 2016).

The first CRISPR HEG reported in yeast demonstrated near perfect (>99%) inheritance over multiple generations, and in multiple strains (DiCarlo et al., 2015). Moreover, this work demonstrated the feasibility of using HEGs with more advanced modifications which had been previously proposed (Burt, 2003; Esvelt et al., 2014). This included carrying a cut-resistant, but functional version of the target gene on the HEG allele, reversing the changes of one drive with another, and split-drives. In a split-drive, one component essential to the drive mechanism is housed on a separate locus, generally by separating Cas9 from its gRNA (**Figure 1C**). This allows the HEG that carries the gRNA to be safely tested, as it will only behave like a HEG in a lab strain that already expresses Cas9 and will not spread in wild populations. The synthetic target sites needed for

earlier nucleases provided similar protection against unintended spread beyond the laboratory. The publication of CRISPR HEGs in yeast demonstrated that CRISPR gene drives are capable of extremely high conversion efficiencies and gave an initial indication that the CRISPR drives do not suffer from the same genetic instability issues seen with previous programmable nucleases. While these results were encouraging, the natural I-*Sce*I HEG also worked extremely well in yeast but failed to reach similar efficiencies in animals. Fortunately, the first CRISPR HEGs in *D. melanogaster* (Gantz and Bier, 2015), *A. stephensi* (Gantz et al., 2015), and *A. gambiae* (Hammond et al., 2016), each used a *vasa* regulatory element and reported inheritance rates over 90%, a massive improvement over the non-CRISPR HEGs. However, each publication also laid bare challenges that could prevent the effective spread of CRISPR HEGs.

Gantz et al. reported the first CRISPR HEG in *D. melanogaster* (Gantz and Bier, 2015). The HEG was inserted in and disrupted the X-linked *yellow* gene, limiting drive to XX females only. Gantz observed loss of function of the yellow gene target in almost all progeny (97%) of gene drive heterozygous mothers. This also occurred in female progeny suggesting that the maternally inherited drive converted the paternally contributed functional yellow allele in the early embryo. However, later publications found substantially lower inheritance rates (76–85%) with a near-identical constructs but including a fluorescent marker (Champer et al., 2017; Xu et al., 2020). It is probable that part of the seemingly super-Mendelian inheritance of the HEG observed in the earlier study (Gantz and Bier, 2015) was due to the maternal "deposition" of the Cas9:gRNA complex without inheritance of the drive expressing allele itself (Xu et al., 2020). The deposited nuclease and gRNA could cause the disruption of the *yellow* target gene in the absence of inheritance of the HEG itself. This could be problematic, as even with high rates of HDR the (repeated) cutting in individuals that did not inherit the drive allele will lead to mutations in the target gene. Moreover, the first HEG reported in *A. stephensi* showed that even when the drive is inherited to serve as an HDR template, deposition can have a negative effect.

Gantz and Jasinskiene et al. reported the development of a synthetic CRISPR HEG in *A. stephensi* (Gantz et al., 2015). Drive inheritance was scored separately in the progeny of males and females that inherited the drive allele from their father, and in the progeny of males and females that inherited the drive allele from their mother. Heterozygous parents of either sex passed along the drive element to 98–99% of their own progeny when that parent inherited the drive allele via the paternal line (drive carrying grandfather). However, strikingly, the maternal contribution of the drive allele (and accompanying maternal deposition) caused germline conversion rates to sharply drop, with only 56% of progeny from males and 62% of progeny from females having inherited the drive element. Moreover, unlike autonomous expression from the drive allele, the maternally deposited nuclease also affected somatic tissues. Maternal deposition was seemingly resulting in nuclease activity early in the embryo when HDR was not favoured, converting the target alleles to resistant alleles that could no longer be converted when expression occurs in the germline and HDR is favoured. Even if deposition-based conversion had been efficient, somatic drive activity has the possibility to cause its own issues. This was most strikingly highlighted by the first *A. gambiae* CRISPR HEG targeting candidate population suppression genes.

Hammond et al. identified three genes that, when disrupted, confer a recessive female sterility phenotype in *A. gambiae* (Hammond et al., 2016). They created Cas9 HEGs in each gene and demonstrated extremely high inheritance rates in both females and males (99%). However, the HEG heterozygous females unexpectedly produced only 0–9% of the number of larvae wild-type females did–a sterility effect that was intended to be limited to the drive homozygotes. They showed that *vasa*2-Cas9 expression was not fully germline restricted, and the nuclease was being expressed in somatic tissues. This lead to nuclease activity in some somatic cells, causing the remaining functional copy of the target gene to be lost, and the recessive phenotype to present in (initially) heterozygote individuals. Additionally, they identified mutations in the female fertility genes that, while preventing Cas9 cleavage, did not disrupt the normal function of the genes. They proposed that depending on the reproductive load of the drive, these functional resistant mutations could prevent the collapse of the target population and demonstrated this in a follow-up publication (Hammond et al., 2017).

Together, this initial set of studies demonstrated that maximising inheritance bias, while minimising unintended fitness costs and the creation of inheritable resistance mutations, remains a challenge with Cas9 based HEGs. Moreover, subsequent studies with HEGs in *M. musculus* (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021), *A. aegypti* (Li et al., 2020; Verkuijl et al., 2020; Reid et al., 2021), *P. xylostella* (Xu et al., 2021), and *Arabidopsis thaliana* (Zhang et al., 2021) have generally proved less efficient than in *Drosophila* and the *Anopheles* mosquitoes. Unintended DNA repair outcomes, inopportune drive expression, and deposition have emerged as the most important impediments for developing efficient HEGs.

3 THE MAIN TECHNICAL CHALLENGES FACING SYNTHETIC HEGS

3.1 Unintended DNA Repair Outcomes

In most cases, the highest possible inheritance biasing rate is desired when developing a HEG. However, target alleles that remain uncut may be converted in the following generations, whereas cut-resistant mutations cannot. As such, the ratio between drive conversion events and unintended outcomes can often be more important than the inheritance biasing rate alone. HEGs may be more susceptible to resistance than other gene drives because DNA damage and repair are directly involved in their inheritance biasing mechanisms. This means a HEG can directly create resistance to itself that was not already present in the target population. This "induced" resistance is in the form of sequence changes to the target allele by unintended DNA repair pathways, often collectively described as non-homologous end

joining repair (NHEJ), that prevent further cutting by the nuclease. Problematically, this resistance, if arising anywhere in the germline lineage, can, in addition to lowering the inheritance biasing efficiency of the drive, also be inherited and contribute a new resistant allele to the population's gene pool.

The consequence of the mutation depends both on the target gene and the nature of the DNA lesion. With "type-1" resistance mutations preventing cutting by the nuclease, but otherwise leaving the function of the target gene intact, and 'type-2" mutations preventing cutting by the nuclease as well as preventing regular function of the gene (Champer et al., 2017). Generally, type-1 resistance mutations are substitutions or small in-frame (n·3bp) insertions or deletions in the exons of protein coding genes. Type-2 resistance mutations are more likely with mutations causing a frame-shift in the exons of protein coding genes and large insertions and deletions. The importance of the amount and type of resistance mutations produced depends much on the particular application of the HEG. For a drive targeting a neutral locus, the distinction between type-1 and type-2 resistance will have no practical significance. In contrast, for a population suppression drive that aims to disrupt a particular (essential) gene, the ratio of type-1 and type-2 resistance alleles produced can be far more important than the overall amount of resistance alleles. Type-2 resistance alleles may slow the spread of the HEG, but they ultimately still contribute to the HEG effector function (disruption of the target gene). In contrast, even extremely rare type-1 alleles may allow for a population to quickly rebound or even be largely unaffected by a suppression HEG (Hammond et al., 2017).

In addition to non-HDR outcomes, incomplete or internal HDR may also be a significant source of mutations. Alleles carrying parts, but not the whole drive element, have been reported in a number of publications studying CRISPR-Cas9 HEGs (Champer et al., 2017; Carrami et al., 2018; Champer et al., 2018; Oberhofer et al., 2018; Pham et al., 2019). In some cases, this can be explained due to internal recombination similar to what occurred with the repetitive ZFN and TALEN HEGs discussed earlier (Simoni et al., 2014). Oberhofer et al. tested a HEG with four gRNAs and found that the repetitive sequences introduced with multiple gRNAs likely caused the drive construct to internally recombine (Oberhofer et al., 2018) (**Figure 2A**). It is not clear if internal recombination is fully independent of the homing mechanism or if a significant fraction of the incomplete drive alleles are created through partial HDR from an otherwise intact donor allele. In some cases, the recovered incomplete drive alleles more strongly suggest they emerged due to partial copying.

In contrast to I-*Sce*I, ZFN or TALENs, Cas9 identifies its target through Watson-Crick base pairing of ≈20 nucleotides of the gRNA with the genomic DNA. Some publications have specifically identified partial drive alleles that are consistent with the gRNA gene target sequence having been used as one of the homology 'arms' during the homing process (Pham et al., 2019; Champer et al., 2017). This results in only part of the drive allele being identified as "missing" from the recipient chromosome, generating a partial copy (**Figure 2B**). However, different repair processes may give the same ultimate product (**Figure 2C**). Internal recombination and partial homing may be more common than is currently recognised. In almost all cases, inheritance rates are determined by scoring the presence of a dominant fluorescent gene linked to the HEG which may also be a partial drive allele (Oberhofer et al., 2018). While in most cases incomplete HDR should result in a type-2 resistance mutation, these partial homing events may pose a problem for drives with distinct cargo genes or sequence changes. Of particular importance, HEGs have been developed that carry sequences that rescue the function of the gene that they disrupt and partial homing of only these rescue sequences may create type-1 resistance alleles.

There may be a set of DNA repair outcomes, such as mitotic recombination and meiotic drive, that are underappreciated because they do not leave a distinct mutational signature. With mitotic recombination, DNA repair causes a dividing cell to produce daughter cells, where one daughter cell has two copies of a paternal chromosome region, and the other has two copies of the maternal chromosome region (**Figure 2D**). The production of individual cells homozygous for a particular parental gene resembles the outcome of homing, however, mitotic recombination does not directly bias the inheritance of any allele as reciprocal cells homozygous for the other allele are also created. However, in one study, *D. melanogaster* females carrying a single copy of the dominant female sterility inducing ovo^{D1} transgene could nonetheless produce viable offspring due to mitotic recombination induced by nos-Cas9 (Allen et al., 2021). Mitotic recombination can seemingly be a substantial outcome of DNA damage, and may be relevant in a HEG with a dominant acting effector such as sex-conversion. Mitotic recombination is most commonly studied by targeting both homologous chromosomes (Brunner et al., 2019; Allen et al., 2021), however it has been demonstrated to occur when only one homolog can be cut (Sadhu et al., 2016).

Finally, if DNA repair fails altogether, the cut recipient chromosome may instead be lost (**Figure 2E**). Loss of haploid cells or fertilised embryos carrying the recipient chromosome will in effect increase the relative inheritance of the donor chromosome, providing a potential separate mechanism of inheritance bias for HEG drives. If the recipient chromosome is marked, inheritance bias through homing or through the loss of the recipient chromosome can be distinguished. An under representation of the recipient chromosome marker has been reported in multiple publications with an element otherwise expected to function through homing (Guichard et al., 2019; Xu et al., 2020; Terradas et al., 2021b), with in some cases meiotic drive seemingly exclusively mediating the observed inheritance bias (Li et al., 2020; Verkuijl et al., 2020). In one study, under representation of a restriction enzyme site nearby the I-*Sce*I cut site on the recipient chromosome was suggested to be due to DNA repair after homing also replacing the nearby marker (termed "co-conversion" or "copy-grafting") (Windbichler et al., 2011). All the pre-CRISPR HEG studies we discussed preformed crosses with marked chromosomes. However, for CRISPR HEGs only a small minority of studies have used a marked recipient chromosome, making it difficult to judge the extent of this phenomenon.

Maximising the efficiency of HDR after a Cas9 induced DNA break is a major topic of research because of its broad applicability to biological and medical sciences (Nambiar et al., 2022). Most research into site-specific HDR has been with an exogenously supplied repair template, and less is known if or what the specific dependencies are for efficient interchromosomal HDR. In addition, many of the interventions that may be used to boost HDR may not be suitable for a gene drive context. Below we will discuss some specific alterations to HEG design that have been investigated in an attempt to steer the number of resistance alleles, the ratio between type-1 and type-2 alleles, and mitigate their effect once they do emerge. The most important of these has been to limit HEG expression to when HDR is more likely, which has a number of additional benefits. However, actually limiting drive activity to this "ideal" window has been challenging.

3.2 Spatial and Temporal Restricted Drive Expression

For a drive to function as a mechanism for super Mendelian inheritance, homing need only occur in the relatively small number of cells that make up the germline lineage (**Figure 3A**). Editing in any other cell lineage forming somatic tissues does not contribute to the drive inheritance biasing rate and in most cases not its effector function. Indeed, "somatic cutting" can be a significant source of additional fitness costs. In the most direct sense, many proposed population suppression drives will perform best when homing is tissue-restricted. These drives rely on heterozygote individuals being unaffected by a particular modification (e.g., disruption of a haplosufficent essential gene), yet passing the drive along at increased rates. This is achieved by restricting homing (and therefore induced homozygosity) to the germline. The effector modification remains heterozygous in tissues where it is required for normal function. An example of this is the *doublesex* targeting *A. gambiae* drive (Kyrou et al., 2018). More generally, for any drive, the unnecessary activity of the drive in somatic cell lineages may contribute to an additional fitness cost of the drive such as from off-target effects (Langmüller et al., 2021). This is compounded by the fact that the ability to perform HDR varies strongly by cell type and on-target resistance mutations that carry a significant fitness cost may be more likely to emerge in cells of somatic lineages.

A prominent hypothesis is that interchromosomal HDR after a DNA break is most likely if the DNA break coincides with meiosis I (Burt and Trivers, 2006; Xu et al., 2017; Champer et al., 2018, Champer et al., 2020 S. E.; Grunwald et al., 2019; Pfitzner et al., 2020; Terradas et al., 2021b; Terradas et al., 2021c; Kandul et al., 2021; Li et al., 2021; Taxiarchi et al., 2021; Weitzel et al., 2021; Xu et al., 2021). During this time chromosomal homologs exchange information through crossing-over events. The alignment of the homologs in the cell and activation of particular DNA repair machinery (Kadyk and Hartwell, 1992; Haber, 2015; Enguita-Marruedo et al., 2019) may make this timing more suited for interchromosomal copying of the HEG. As such, almost all synthetic HEGs have been designed to be active in the germline by flanking the nuclease transgene with the putative promoter, 5′UTR, and 3′UTR sequences of an endogenous germline specific gene (**Figure 3B**). The most widely tested have been sequences from the *nanos* and *vasa* genes.

There are a number of examples where the locus from which the HEG components are expressed seems to affect otherwise identical drive elements (Chan et al., 2013a; López Del Amo et al., 2020a; Grunwald et al., 2019; Reid et al., 2021), (Champer et al., 2019a) compared to (Champer et al., 2017; Champer et al., 2020 S. E.). One potential major challenge of achieving restricted expression is that a HEG inserted into an endogenous gene may be influenced by that gene's cis-regulatory elements and broader chromatin context (**Figures 3C,D**), which under normal circumstances facilitate the specific expression pattern of the target gene. Ironically, for drives inserted into essential genes these regulatory elements may prime the HEG to express in the cells that drive activity would be most undesirable. This in effect, can make that locus one of the worst possible places for the HEG to be inserted to prevent "leaky" expression coinciding and interfering with the target gene's activity. Split-drives, where the Cas9 is expressed at an unrelated locus, can avoid this regulatory element mismatch, but this may also cause them to be behave differently if reconstituted to a single element drive (Terradas et al., 2021a). The challenge of avoiding leaky expression is further compounded by some enhancers and other regulatory elements being located in the coding region of genes (Birnbaum et al., 2012). The presence of such elements in the target gene, and the absence of those elements from the germline genes, could alter the expression of Cas9 away from the germline restricted expression pattern the HEG is trying to recapitulate. Finally, the regulatory components of other drive components (e.g., fluorescent marker or cargo genes) may also interfere with the intended expression pattern of the nuclease and gRNA genes (Champer et al., 2019a).

Somatic cutting, in the absence of maternal deposition, has been reported for many Cas9 expression regulatory elements (Gantz et al., 2015; Champer et al., 2018; Kandul et al., 2020; Li et al., 2020; Verkuijl et al., 2020). As described above, this is generally detrimental, however, there are a limited set of cases where somatic conversion can be an intended part of the drive effector mechanism. Carrami et al. aimed to develop a sex-conversion drive disrupting the autosomal transformer (*tra*) gene (Carrami et al., 2018). By deliberately selecting promoters that would be active in somatic tissues, homozygous disruption of *tra* by the drive and somatic mutations would, in the medfly, convert XX females into fertile males (Pane et al., 2002). They performed their experiments in *D melanogaster* in which *tra* disruption leads XX individuals to develop into infertile pseudomales. However, in practice the XX individuals displayed an intermediate intersex phenotype (and were infertile). Males were unaffected by the somatic disruption of *tra* and displayed modest estimated homing rates (≈56%) in their germline. This work highlights that even in cases where somatic activity is desired, achieving a uniform disruption of the target gene in all cells can be a challenge. The outcome of such intermediate conversion, with some cells converted and others not, is called mosaicism and in many reports this is how somatic HEG activity presents.

While theoretically somatic and germline activity can be fully distinct processes, in some cases, the drive activity that gave rise to the somatic conversion did not necessarily (only) happen in the wrong

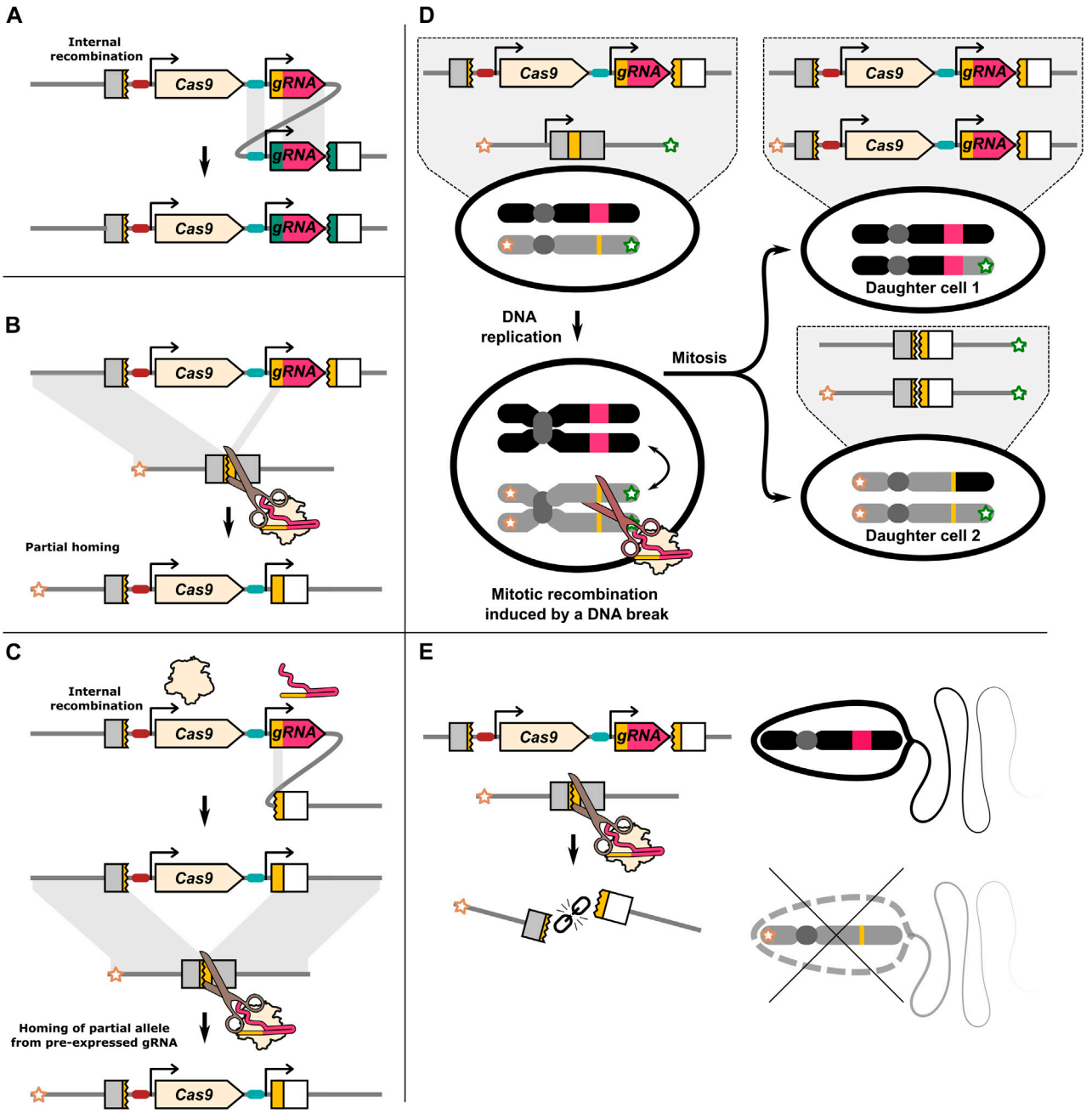

FIGURE 2 | Illustration of DNA repair outcomes that may be associated with a HEG. **(A)** Repetitive sequences within the HEG may lead to internal recombination. The gRNA promoter and the constant gRNA "backbone" sequence in multiplexing drives may be particularly susceptible to internal recombination. **(B)** Partial homing by means of the gRNA target sequence located on the drive allele. The gRNA gene contains sequence homology to its target. This may allow for partial homing. **(C)** Recombination of the gRNA target sequence with a partial target sequence adjacent to the drive element. If the resulting allele homes it may resemble the product of partial homing even if a chromosome marker is present. **(D)** Cas9 induced DNA breaks have been reported to lead to mitotic recombination. This can produce two daughter cells that have loss of heterozygosity. Daughter cells generated by mitotic recombination can under some circumstances resemble products of homing. **(E)** In some cases a DNA break can lead to the loss of the target/recipient chromosome. This can result in inheritance bias through meiotic drive.

cell lineages, but also at the wrong developmental time. Drive activity in the early embryo can convert cells that go on to give rise to both the germline and somatic tissue, producing the associated somatic phenotype later in development. In these cases, preventing leaky expression early in development may simultaneously lower the production of resistance alleles (if HDR is indeed not favoured) and decrease disruption of the target gene in somatic tissues.

Another potential source of resistance mutations occurs at the other end of differentiation, post meiosis. Any recipient chromosomes that escaped cutting in the germline will be

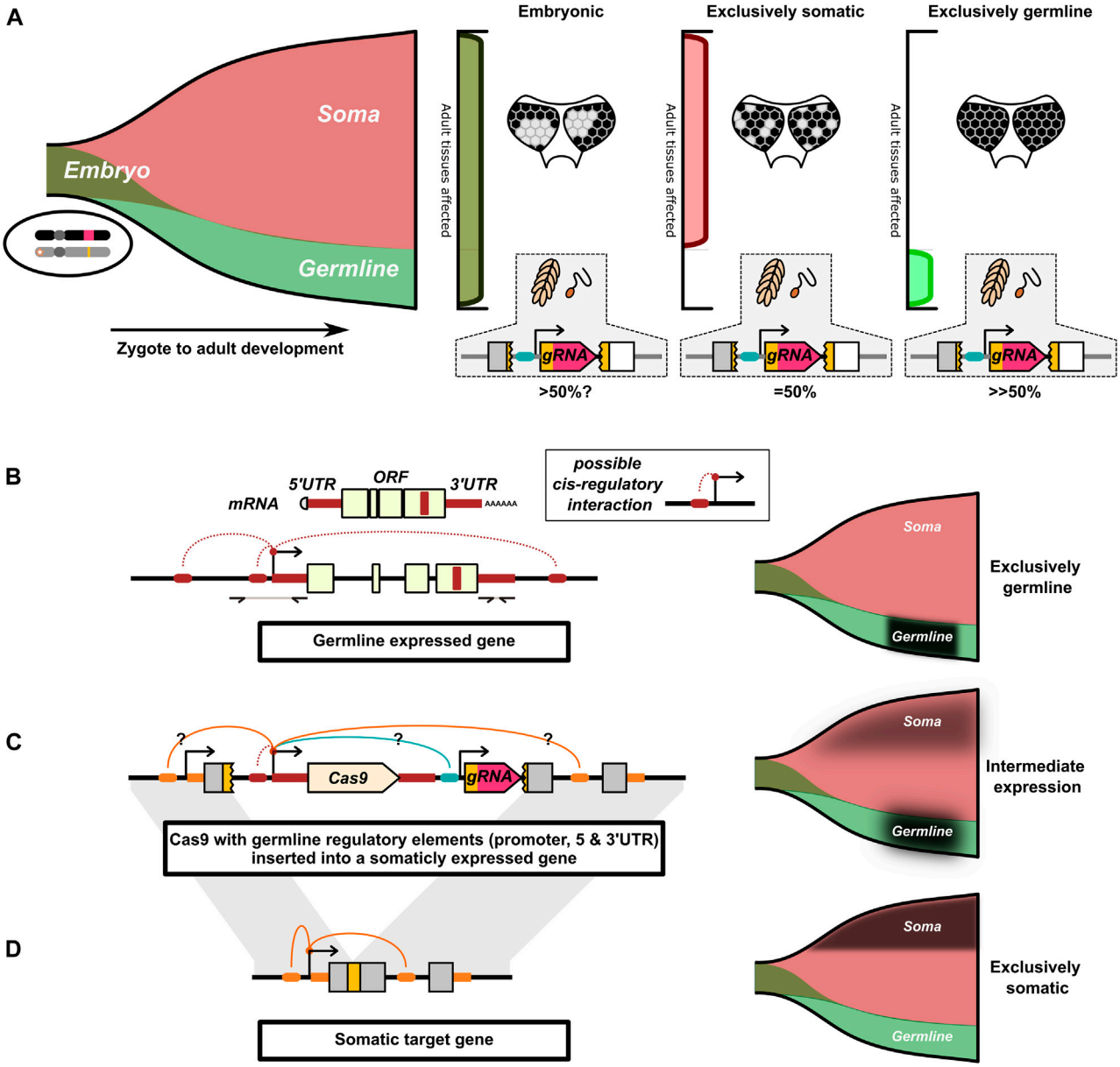

FIGURE 3 | Illustration of the interaction of spatial and temporal restricted drive expression. **(A)** Drive activity in the early embryo can simultaneously affect cells of the somatic and germline lineages. Drive activity later in development can independently affect the germline and somatic cells. The funnel represents the cells that compose an individual as it develops from a zygote to an adult (left-to-right). Early in development, cells are simultaneously part of both the germline and somatic cell lineages. Only later in development do the germline and somatic cell lineages diverge. The pattern of somatic mosaicism in adult tissue can under certain circumstances be indicative of the timing of cutting, here indicated by different patterns of loss of pigment in the eye. In these cases, the later in development the somatic gene is disrupted, the more fine-grained the mosaic pattern. **(B)** Cas9 can be engineered to mimic the expression of endogenous germline restricted genes. This is commonly approached by use of the promoter, 5′and 3′ UTR of a germline restricted gene. The 5′ and 3′UTR can be identified from mRNA transcripts. Generally, a few Kb of sequence upstream of the 5′UTR are taken to capture the putative promoter. We have indicated additional speculative cis-regulatory interactions important for germline restricted expression that are not captured by this approach. **(C)** The Cas9 gene with the germline regulatory elements is inserted into the target gene's locus. Cas9's expression may be affected by cis-regulatory interactions with the target gene and with other drive components. We speculate that this may result in the Cas9 gene taking on an intermediate expression pattern resulting from the combination of different cis-regulatory interactions. **(D)** The target gene commonly has a somatic expression pattern that may not be conducive to homing. For (B–D), the proposed expression pattern of the respective allele is indicated by a black overlay on the funnels.

separated from the donor chromosome once meiosis has occurred. If transcribed or translated drive components persist, cutting may occur after this point, resulting in repair by interchromosomal HDR being impossible. DNA damage near or post-meiosis may therefore be a source for resistance alleles (Champer et al., 2017; 2019a).

It is currently not clear if significant cutting occurs after meiosis. However, that expressed/translated HEG components persist into haploid cells that do not contain the HEG genes has been established for many drives. This is because the HEG components can go on to affect the fertilised zygote. This is the phenomenon of deposition we introduced earlier, and it has similar consequences to that of early embryonic leaky expression. However, there are important differences between the two processes.

3.3 Parental Effects (Deposition)

Many publications studying HEGs have noted that genetically identical individuals will show different somatic phenotypes and inheritance biasing efficiencies depending on which parent contributed the Cas9 and gRNA genes (Champer et al., 2017, 2018, 2019a; Carrami et al., 2018; Oberhofer et al., 2018; Guichard et al., 2019). These types of parental effects have even been observed in individuals that did not inherit any genetic components of the drive, indicating the deposition of already expressed drive components. In almost all cases, an exclusive maternal effect is observed, where a female carrying the HEG transgene(s) is thought to contribute the gRNA and/or nuclease protein/mRNA to her haploid eggs. While these parental effects are commonly referred to as deposition, it is important to note that for some HEGs an alternative or additional mechanism such as imprinting has not necessarily been excluded.

A key difference between "leaky" embryonic expression and embryonic cutting by deposition is that in the case of deposition, cutting can occur in the absence of inheritance of the drive. As such, even if interchromosomal HDR were favoured, deposition may result in the target allele being cut when not paired with a HEG allele. In addition, the activity of the deposited drive components may be expected to be early in development, affecting both the somatic and germline cell lineages. Cas9 protein half-life in cells and embryos has been estimated to be (substantially) less than 24 h (Kim et al., 2014; Burger et al., 2016). In *Drosophila*, the first meiotic divisions occur in third instar larvae (> 2–days) in males, and early pupal stages (> 3–days) in females (Hartenstein, 1993). This suggests that if Cas9 protein stability is limited to hours, it would not persist long enough to overlap with meiosis I in many species. Moreover, even if Cas9 protein persists to when meiotic divisions occur, most target alleles may already have been cleaved earlier in development. The activity window of deposited Cas9 mRNA is harder to predict. The mRNA's translation in the embryo would likely delay and extend Cas9's window of activity, while 5′ and 3′UTR sequences in the Cas9 mRNA copied from a germline gene may specifically limit the timing and location of translation. Moreover, it is possible that if mRNA deposition were to occur, the translated Cas9 would only become active in progeny that inherited the gRNA expressing gene, as the gRNA is highly unstable when not in complex with Cas9 protein (Hendel et al., 2015; Ma et al., 2016; Wang et al., 2019).

In general, deposition can be a substantial issue for effective homing, but it should be noted that the fitness costs associated with deposition-induced drive activity may be less detrimental than that of equivalent leaky expression drive activity (Beaghton et al., 2019). This is because deposition affects the progeny independently of if they have inherited the drive or not. In contrast, the fitness cost of leaky expression is limited to those individuals carrying the drive element. However, the possibility of creating inheritable (type-1) resistance mutations in non-drive inheriting progeny may still make deposition substantially more problematic.

Isolating and quantifying the resistance allele contribution of deposition can be difficult. Maternal deposition is most readily identified by disruption of the paternally contributed allele in the absence of inheritance of the drive allele. However, the maternally contributed allele may have been disrupted at the same time, or instead at any point in the mother's development. As such, it is not possible to directly distinguish between germline resistance mutations and those that arise in the early embryo due to deposition; but there are two key pieces of evidence that may suggest a particular timing.

Firstly, particular DNA lesions have been found repeatedly in multiple offspring from the same parent (Champer et al., 2017, Champer et al., 2018; López Del Amo et al., 2020a). This can suggest that the specific mutation arose in the HEG drive parent's germline, was replicated by cell divisions, and was passed along to multiple offspring. Interestingly, the fraction of offspring inheriting the same mutation may provide evidence for when it occurred in the parents germline (López Del Amo et al., 2020a). However, it should be noted that while NHEJ mutations are variable, their scope can be heavily influenced by the sequence context of the DNA break increasing the likelihood of identical mutations arise independently (Allen et al., 2019; Chakrabarti et al., 2019). Nonetheless, statistical analyses can detect if particular mutations co-occurred more in progeny of the same parent than between individuals with different parents (Champer et al., 2018).

The second observation is mosaicism. If there is a mix of mutant alleles found within an individual progeny, this indicates they arose after the first genome replication and did not arise in the parent's germline. However, this analysis only works if the experiment is performed in such a way that the paternally contributed allele is cut-resistant and cannot in itself generate a mosaic outcome. As a caveat to this, Chan et al. has suggested that a mosaic phenotype observed in progeny that did not inherit their I-*Sce*I HEG allele was due to the inheritance and replication of an unrepaired DNA break instead of parental deposition (Chan et al., 2011). However, this was not further investigated.

Champer et al. reported that for a single drive element (comprising both *nanos*-Cas9 and a gRNA), the degree of embryonic cutting they saw in progeny subjected to maternal deposition and inheriting the drive was similar in the cases where the mother was heterozygous or homozygous for the drive (Champer et al., 2017). They offered that this "implies that most maternal Cas9 persisting to the [progeny's] embryo stage was expressed after drive conversion events" in the mother. This was further supported by evidence that the rate of embryonic cleavage from deposition was lower when drive conversion was less likely, such as when mothers carried mostly resistance alleles instead of an additional wild-type target allele. Interestingly, in

the progeny from these crosses the fraction of progeny with a somatic phenotype was similar regardless of whether a drive allele was inherited or not. This implied that most Cas9 persisted through to the embryo after maternal expression in diploid cells, rather than being expressed after meiosis and correlating with drive inheritance. These experiments provide some insight into the complex ways in which deposition can manifest for a particular expression pattern. In addition, it should be noted that deposition, or its interpretation, can be context dependent, as is well illustrated by the *zpg*-Cas9 *A. gambiae* drive. Crosses with maternal (Fuchs et al., 2021), paternal (Kyrou et al., 2018), or no deposition (Hammond et al., 2021a) have all been described for this same drive. Moreover, in *Drosophila* it has been reported that the embryo resistance rates can be strongly affected by the background genetics of crossed individuals (Champer et al., 2019b).

As introduced earlier, early embryonic nuclease activity associated with maternal deposition can cause a drive to affect somatic tissues (with the accompanying issues described above). While DNA repair associated with deposition-based cutting seems to be more error-prone, interchromosomal HDR does occur. The use of split-drives has allowed deposition to be investigated in more detail. Many HEGs have been tested in the form of a split-drive, where the Cas9 is located at an unlinked locus. Generally, this results in 50% of progeny inheriting the Cas9 gene independent of inheritance of the main drive allele carrying the gRNA(s). A number of publications have noted that individuals that only inherit the gRNA element can nonetheless pass it along at increased rates and this has been termed "shadow drive" (homing through deposited factors) (Champer et al., 2019a; Guichard et al., 2019; Kandul et al., 2020; Terradas et al., 2021b) (**Figure 4**). The activity window of deposited nuclease may be expected to be early in development with limited persistence. As such, examples of efficient shadow drive may provide a counter point to the hypothesis that homing is limited to meiotic cells that emerge later in development. Important in this interpretation is that deposition patterns can seemingly differ for the nuclease, gRNA, and Cas9:gRNA complex.

Kandul et al. created a split-drive in *D. melanogaster* targeting the *white* gene, and carrying a gRNA targeting the *yellow* gene in trans (Kandul et al., 2019). They tested four regulatory elements expressing Cas9 from a separate locus, and mediated significant inheritance bias with an average estimated homing rate of 73%. Interestingly, when the nuclease was carried by the grandmother, the estimated homing rate in the parent's germline was roughly the same (69%), even when the Cas9 gene had not been inherited. Shadow drive through maternal deposition with the four Cas9 regulatory elements they tested was seemingly just as efficient at biasing the inheritance of the gRNA HEG element as germline expression of the nuclease was. However, in these first crosses the gRNA gene was contributed by the grandfather, providing no opportunity for the Cas9:gRNA to complex before being deposited. In a subsequent cross with the grandmother carrying both the Cas9 and gRNA genes, the estimated homing rate dropped sharply to 9.2% in trans-heterozygotes (gRNA + Cas9) and to 6% in heterozygotes (gRNA only). When both Cas9 and gRNA were maternally deposited, cleavage could occur in the early embryo, forming resistance alleles, which then prevented drive conversion at a more opportune stage. This did not occur with maternal deposition of only Cas9 (not gRNA) into an individual that can nonetheless express the gRNA. gRNA expression, thought to be constitutive, and subsequent complex formation with the deposited Cas9 protein seemingly limited cutting to a more opportune stage for inheritance bias even in the absence of Cas9 expression. A similar result was reported by López Del Amo et al., with Cas9 and gRNA carrying mothers having a detrimental effect on inheritance bias by their progeny while contribution of the gRNA from the father and Cas9 from the mother did not (López Del Amo et al., 2020a).

4 WHAT STRATEGIES DO WE HAVE TO COMBAT THESE CHALLENGES?

4.1 Achieving Restricted Nuclease Expression

Restricting nuclease activity to cells and developmental stages where HDR is expected to be favoured and somatic tissues are unaffected is an optimisation strategy commonly pursued within the field. This is especially important in population suppression drive systems which rely heavily on the fitness of drive-carrying heterozygous (female) individuals (Eckhoff et al., 2017; Beaghton et al., 2019; North et al., 2020; Champer et al., 2021a,b). To achieve this restricted activity, the field has largely relied on identifying and testing multiple genes which are predicted to have the desired expression/activity profile. The putative regulatory sequences of these genes are then isolated and used to express Cas9. This strategy has shown success such as the improvements achieved by *zpg* expressed Cas9 in *A. gambiae* compared to Cas9 expressed with *vasa*2, *nanos*, or *exu* (Hammond et al., 2021a). This change of expression resulted in the reduction of both somatic drive activity and deposition of the nuclease while maintaining the inheritance biasing efficiency. While this trial-and-error approach has yielded improved HEGs in multiple species, insights into what underlies any improvement in performance are very limited as many changes are made simultaneously that cannot be deconvoluted.

The future design of HEGs may be aided by studying the effect of "stacking" multiple limited regulatory mechanisms. In addition to the use of promoter/5′UTR and 3′UTR sequences, other endogenous regulation mechanisms can be included, such as tissue-specific splicing (Salles et al., 2002; Tsujimoto et al., 2013; Sutton et al., 2016), modulation of protein degradation (Chassin et al., 2019), sub-cellular localisation (Goeckel et al., 2019), and inclusion of miRNA binding sites (Loya et al., 2009). Ideally each regulatory system should make as limited and well defined a change as possible. Decoupling of expression timing from expression levels may be a useful first candidate as the stacking of regulatory mechanisms may be expected to cause a cumulative decrease in activity levels due to imperfect removal of inhibition. Grunwald et al. demonstrated this principle with a

HEG by using a strong and constitutive promoter to express Cas9 that could only be translated once a stop codon had been excised by separate and germline-restricted expression of a recombinase (Grunwald et al., 2019). An important downside of Grunwald's approach was that once activated, the Cas9 could no longer be shut-off. A similar approach, but using a transcription factor intermediate such as the GAL4–UAS system would allow for reversible activation (Fischer et al., 1988). Such a system may particularly benefit "integral" gene drives.

Integral gene drives make direct use of an endogenous gene for their expression (Nash et al., 2019). Hoermann et al. demonstrated, in three different genomic loci of *A. gambiae*, that an artificial intron can be used to express both an effector protein and the host gene product (Hoermann et al., 2021). A gRNA targeting the unmodified locus was also included, which allowed for efficient inheritance bias of the whole element with a separately expressed Cas9. Inclusion of the large Cas9 gene (> 4 Kb) in an integral gene drive context has been recently demonstrated in mice, where Cas9 was integrated at the very end of the coding region of the *Spo11* gene (Weitzel et al., 2021). This design resulted in Cas9 being co-translated with the endogenous gene, with the aim of restricting Cas9's activity to match that of *Spo11* which is involved in meiotic recombination. Using this approach, Weitzel et al. demonstrated for the first time homing in male mice despite earlier efforts with other regulatory systems (Grunwald et al., 2019; Weitzel et al., 2021). However, the majority of target alleles remained uncut, indicating that improvements in regulating the timing of Cas9 activity came at the cost of its activity level. An intermediate amplifier of expression may address these expression issues and, if smaller than Cas9, interfere less with the endogenous gene's function (the endogenous *Spo11* gene was impaired by the Cas9 insertion).

Finally, there may be specific interventions that can address the effect of Cas9 deposition. In a study in *A. gambiae*, the I-*Ppo*I homing endonuclease was expressed by the testis-specific promoter *β-tubulin* to establish a synthetic sex ratio distortion system by shredding the X chromosome in the paternal germline (Windbichler et al., 2008). However, no viable embryos were produced because paternally deposited I-*Ppo*I also shredded the maternally contributed X chromosome in the zygote. A subsequent study was carried out to reduce the half-life of the endonuclease by systematically introducing point mutations into the protein (Galizi et al., 2014). Strains with high levels (95–97.4%) of male-biased sex distortion and fertility rates similar to controls were eventually generated using this approach. Interestingly, the modified I-*Ppo*I was recently combined with a HEG drive system into a "sex-distorter gene drive" (Simoni et al., 2020). When tested at three new loci, expressed with the identical *β-tubulin* promoter, male sterility was reestablished to varying degrees, presumably due to locus-dependent changes in expression of the I-*Ppo*I endonuclease, causing sufficient protein to persist into the embryo. By introducing a 100-bp GC-rich DNA sequences into positions -271, -244, and -355 upstream of the start codon, respectively, transcriptional activity of the *β-tubulin* promoter was reduced to 0.5, 8.1, and 16.2%. The promoter variant with 8.1% transcriptional activity, coupled with a destabilised I-*Ppo*I, was inserted into the *dsx* locus and was found to have no detectable sterility in drive heterozygous males. Similar approaches may also address deposition of Cas9 in a HEG context.

4.2 Multiplexing

The targeting of multiple sequences ("multiplexing") has been proposed as a means of addressing one of the most significant impediments to HEG drives - resistance (Esvelt et al., 2014). If an initial attempt at homing fails and induces a mutation, multiplexing may still allow for homing through cleavage at an alternate cut site. Moreover, multiplexing would also allow the HEG to drive in individuals that have preexisting sequence variation in a subset of cut sites. Another benefit, specifically when targeting high-fitness cost genes, is that for complete resistance (resistance at all target sites) more extensive sequence changes would need to occur and this reduces the likelihood of the formation of type-1 resistance mutations. In terms of feasibility, multiplexing is particularly convenient with CRISPR-Cas9 nuclease as it only requires expressing additional gRNAs. Under these assumptions, computational modelling has indicated multiplexing to be an effective strategy to reduce the formation and accumulation of resistant alleles (Marshall et al., 2017; Noble et al., 2017; Prowse et al., 2017; Champer S. E. et al., 2020; Edgington et al., 2020). However, some practical challenges have emerged with this "classical" multiplexing approach.

In the classical approach to multiplexing, multiple gRNAs targeting closely linked adjacent sequences of a single gene are expressed in a single drive transgene (**Figure 5A**). Additional sequences need to be removed or replaced on the donor chromosome to prevent the HEG from cutting itself at these additional sites. For a single target HEG, the ends of the cut site can be perfectly homologous to the donor chromosome. However, in a multiplexing system, any individual cut site can no longer generate two DNA strands that are perfectly homologous to the donor chromosome (**Figure 5B**). There are indications that these extraneous, "unmatched" sequences could reduce the homing efficiency (Champer et al., 2018, Champer et al., 2020 S. E.; López Del Amo et al., 2020a), presumably due to the additional resection that would not need to occur prior to HDR with a perfectly homologous repair template (Liu and Kong, 2021; Ang et al., 2022). López Del Amo et al. introduced 20bp truncations in the homology arms either side of a *D. melanogaster* HEG (López Del Amo et al., 2020a). These truncations result in 20bp of unpaired sequences on the recipient chromosome that would normally be homologous to the sequence directly adjacent to the HEG. The inheritance biasing rate of the HEG was significantly reduced with truncation on both sides of the HEG. Consistent with this, two HEGs, each with four gRNAs, targeting sites spread over a large region (> 2 Kb) (Oberhofer et al., 2018) performed worse than similar drives with one gRNA (Champer et al., 2017) or two gRNAs targeting a smaller region (Champer et al., 2018). Moreover, additional gRNAs may compete to complex with a limited amount of Cas9 protein lowering the cut rate at any one site (Champer S. E. et al., 2020). These results indicate that any individual cut site in a classical multiplexing may be less efficient than a drive element optimised for only one cut site.

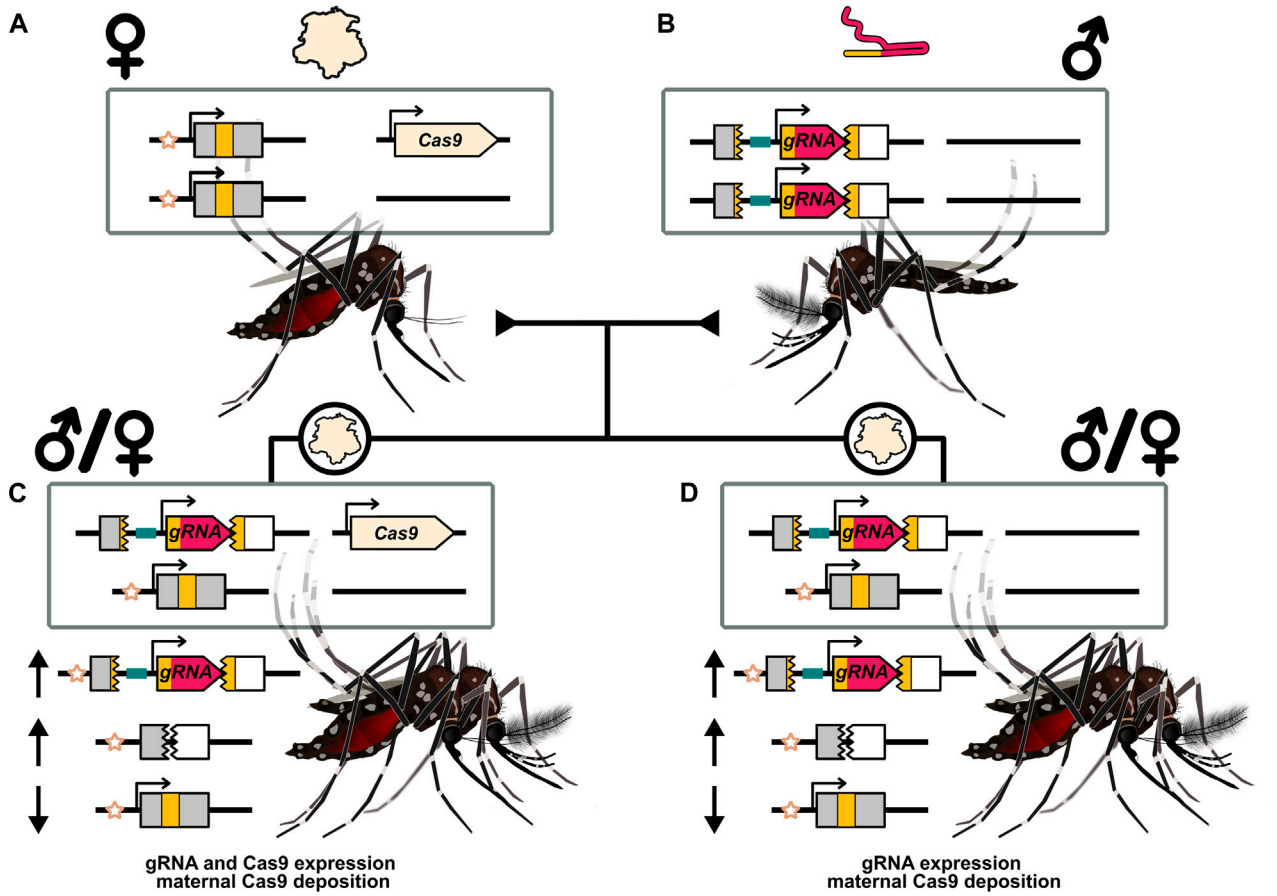

FIGURE 4 | Illustration of maternal deposition and shadow drive in the context of a split-drive. **(A)** A female carrying a Cas9 split-drive element and depositing Cas9 protein into her eggs. **(B)** A male that carries the gRNA drive element and does not deposit the expressed gRNA. **(C)** Progeny of either sex that inherited the two transgenes from each parent separately. Expression of the Cas9 and gRNA genes can allow for inheritance bias. **(D)** Progeny of either sex that inherited the gRNA gene from their father but not the Cas9 gene carried by their mother. Maternal deposition can nonetheless provide a source of Cas9 that can complex with the expressed gRNA and mediate inheritance bias of the gRNA drive element. This is termed shadow drive. The recipient chromosome marker (star symbol) is used to indicate the target alleles that have been replaced by the drive element. Inheritance biasing mechanism of drive expression and/or deposition could operate through a non-copying mechanism. The arrows indicate a relative increase (upward arrow) or decrease (downward arrow) of the particular allele. While mosquitoes are illustrated Ramírez (2019a,b), these results have primarily been documented in *Drosophila*.

Nonetheless, additional studies have indicated that multiplexing can increase the overall efficiency of a HEG albeit in some cases with diminishing returns for additional gRNAs (Champer et al., 2018, Champer et al., 2020 S. E.; Yang et al., 2021), and (López Del Amo et al., 2021) compared to (López Del Amo et al., 2020b).

A novel challenge introduced by multiplexing is expressing different gRNAs simultaneously at similar concentrations without introducing repetitive sequences. Multiple strategies have been proposed to achieve this, many of which involve the excising of individual gRNA from a single long transcript such as with tRNAs (Port and Bullock, 2016; Knapp et al., 2019). However, these excising approaches are frequently not perfectly efficient or leave scars in the form of additional nucleotides attached to the gRNA that can reduce their activity. A separate approach that may be effective is for each gRNA using a set of different (minimal) promoters that have been characterised to have similar expression levels (Anderson et al., 2020). Using different experimentally validated "backbones" for the non-targeting sequences of the gRNA may further reduce the likelihood of internal recombination (Noble et al., 2019).

Cas9 is known to remain bound to its target even after making a double-strand break (Sternberg et al., 2014), and DNA repair occurs more slowly than with other sources of DNA damage (Brinkman et al., 2018). This may provide an opportunity for DNA breaks to occur at different sites before DNA repair is completed, leading to frequent deletions between independent target sites (Brinkman et al., 2018), as has been observed for with some multiplexing HEGs (Champer et al., 2018; Oberhofer et al., 2018; Champer S. E. et al., 2020). In addition, CRISPR-Cas9 has been found to frequently induce large deletions (> 250) at single cut sites (Adikusuma et al., 2018; Kosicki et al., 2018; Nambiar et al., 2022). Large deletions, or simultaneous cutting of at least the outermost target sites of a multiplex drive, could remove all

unmatched sequences from a HEG recipient chromosome, potentially restoring homing efficiency to the level of a single target drive. However, this would also negate some of the benefits of multiplexing, as it may cause the simultaneous loss of all gRNA recognition sites on the chromosome. Recently, a separate drive or multi-locus multiplex strategy has been proposed that may avoid some of the diminishing returns of classical multiplexing.

Multi-locus multiplexing consists of multiple 'parallel' single-target HEGs generated as separate lines, each targeting an adjacent site in the target gene (**Figures 5C,D**) (Edgington et al., 2020). Compared to the classical multiplexing strategy, the separate drive approach will be logistically more onerous but benefits from not being able to generate deletions of all target sites by simultaneous cutting. However, experimental validation of this approach has yet to be reported.

4.3 Targeting Essential Genes

The targeting of essential genes with HEGs has been reported in many studies, but depending on the goal of the system (i.e, suppression or modification), the approaches may diverge. For a suppression drive, the goal is generally to disrupt the essential gene in individuals of the target population. To achieve this, a functionally constrained sequence can be targeted such that few, if any, cut-resistant mutations generated will have a fitness advantage over the drive allele. In *A. gambiae*, an ultra-conserved region of a female-specific isoform of the *doublesex* gene was targeted to impede formation of resistant alleles (Kyrou et al., 2018). HEG drives based on this target site in three cage trials were able to cause complete population crash, and no type-1 resistance alleles were recovered (Kyrou et al., 2018; Simoni et al., 2020; Hammond et al., 2021b). A similar drive, targeting an ultra-conserved exon in a different gene did lead to the emergence of resistance to the HEG in the form of a single nucleotide silent mutation (Fuchs et al., 2021). The target site to which no resistance emerged was located at an intron-exon junction, potentially making mutations liable to disrupt crucial mRNA secondary structures.

Targeting an essential gene can also be used for non-suppression drives as a general approach for lowering the viability of mutations ("home-and-rescue"/rescue HEG). By providing a rescue sequence within the HEG drive construct (**Figure 6A**), resistance can be mitigated as the HEG drive will now have a fitness advantage over type-2 resistance alleles. The role of the rescue sequence is well illustrated by the first HEG drive developed in *A. stephensi*. This drive was inserted into an eye pigmentation gene, *kmo* (Gantz et al., 2015), which was later found to have a recessive fitness cost in females (Pham et al., 2019). This reduced fitness, coupled with a reduction in inheritance biasing efficiency in individuals experiencing maternal deposition caused the drive to fail to reach fixation when released at a 1:10 drive:wild-type ratio and even performed poorly at a 1:1 ratio (Pham et al., 2019). A new version of the HEG was developed that included recoded parts of the *kmo* gene resulting in the drive allele no longer disrupting its function. A subsequent cage trial demonstrated this improved version of the HEG could effectively spread and reach fixation (Adolfi et al., 2020). In addition to biasing its inheritance by cutting target alleles, the improved HEG could also increase in frequency by positive selection when the frequency of type-2 alleles accumulated in the population.

The most common approach for rescuing the function of the target gene is providing a "recoded" version of the sequence the drive allele is disrupting (**Figure 6B**). Like type-1 resistance alleles, these are sequence changes (such as the swapping of synonymous codons) that prevent recognition by the gRNA but leave the target gene functionally intact. While in some cases a single nucleotide substitution can be sufficient to prevent Cas9: gRNA binding, much more extensive recoding is performed to reduce the risk of partial homing (**Figure 6C**). Recoding is further complicated by the need to include noncoding sequences such as the 3′UTR, for which no straightforward synonymous sequence substitution rules exist. In the case of the *kmo* targeting *A. stephensi* drive, the 3′UTR from the *A. gambiae kmo* gene was used in the rescue element (Adolfi et al., 2020).

The manner in which positive selection is conferred to the recoded rescue allele is similar to that expected in a Cas9-based toxin-antidote system where Cas9-induced mutations cause lethality or sterility allowing a cut-resistant rescue/antidote gene to spread in the population (Oberhofer et al., 2019; Champer et al., 2020a). Moreover, deposition can increase the effective inheritance rate of the drive by culling individuals that have inherited a type-2 allele from their drive carrying mother and a functional target allele from their father. Disruption of the paternally contributed target allele by maternally deposited nuclease can make these individuals no-longer viable. Progeny that inherited the drive allele will be protected as they carry the (dominant acting) recoded antidote. However, these drive inheriting individuals will likely have severely reduced homing rates. These rescue HEG systems have been reported in several studies targeting haplolethal (*RpL35A* (Champer et al., 2020b)) or haplosufficient (*rab5*, *rab11*, *spo11*, *prosalpha2*, and *PolG2* (Terradas et al., 2021b; Kandul et al., 2021)) genes. All three of these studies showed increased inheritance of the rescue alleles to varying degrees and demonstrated this strategy to be successful in mitigating the negative effects of type-2 alleles to the drive system.

In the study by Champer et al., multiplexing (two gRNAs) was combined with targeting the haplolethal *RpL35A* gene (Champer et al., 2020b). When the target gene is haplolethal instead of haplosufficent, a single rescue gene may not be sufficient to protect from the lethal effects of deposition. This makes deposition a much more substantial hurdle for these types of systems. Shadow drive may theoretically be expected to create viable progeny by homing an inherited rescue element or a type-1 resistance allele. However, rescue through shadow drive may be very unlikely as deposition often results in mosaic outcomes. Depending on the target gene, any individual progeny may have a significant proportion of cells that have not been rescued by shadow drive and therefore nonetheless become inviable ("lethal mosaicism"). Similarly, with a haplosufficient target, individuals inheriting a maternally contributed type-2 resistance allele may not be rescued by mosaic type-1 alleles produced by deposition-induced cutting.

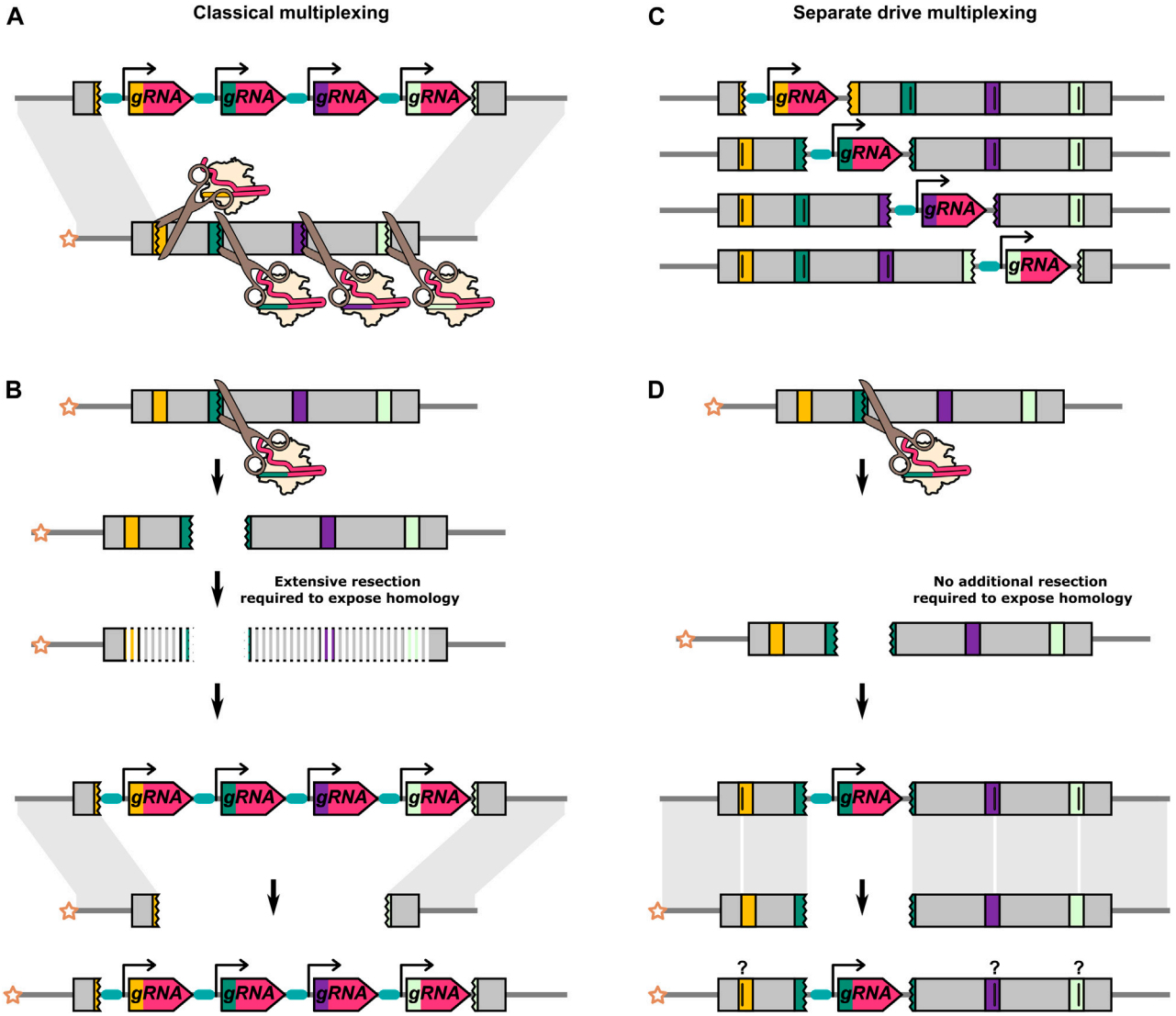

FIGURE 5 | Differences between classical multiplexing and separate drive multiplexing. **(A)** Classical multiplexing construct targeting adjacent sequences within a single gene. Four gRNAs are encoded from a single construct. **(B)** When the outermost gRNAs do not cleave the recipient chromosome simultaneously, the cut caused by any one of the gRNAs will inevitably result in a region of extraneous sequence on the recipient chromosome which is "unmatched" to the donor chromosome. As further 5′-3′ and 3′-5′ resections have to occur prior to HDR, this might reduce HDR efficiency and favour NHEJ. **(C)** Four separate drive elements targeting adjacent sequences within a single gene. These are independent modifications of the same target gene and are not present in the same individual. Alternatively, separate drive constructs could be used to target multiple loci at distinct sites within the genome. **(D)** The cut caused by the gRNA on the recipient chromosome is repaired by using the "matched" homology arm on the donor chromosome as its template. The separate drive elements can include recoded target sequences for the other gRNAs to prevent cutting between elements. It is unclear if and how these types of sequence changes would affect homing efficiency. In A, one gRNA target is shown to be cut in the opposite orientation to the others. The different orientation is to do with the asymmetrical position of the cut site within the standard 20bp gRNA binding site (17bp// 3bp). After a cut with a single gRNA, one end of DNA break will have at least 17bp of homology with the gRNA gene, and the other end will have at least 3bp. With a multiplex design, the outermost gRNAs can be oriented opposite to each other such that both DNA ends carry either the smaller or the larger region of homolgy to the gRNA gene. A DNA end with only 3bp of homology to the gRNA gene will presumably minimise the risk of partial homing/internal recombination. In contrast, if resection after a single cut (see B) is to be minimised, the opposite gRNA orientation may be desirable. For gRNA target sites that are not in the outermost position, there may be no overall "optimal" orientation as it varies with the particular combination of gRNA targets that have cut.

5 CONCLUDING REMARKS

Synthetic homing endonuclease gene drives have been actively researched for over a decade. In this time, the field has developed and characterised a range of designs and applied these to a diverse set of species. Insects, and specifically *Drosophila* and the *Anopheles* mosquitoes, have so far proved substantially more amenable to HEG mediated inheritance bias than other animals and plants. Additional work will prove if the optimisation approaches developed in these insects can be successfully applied in these other species. This effort may be aided by a more systematic and high-throughput HEG test and design approach.

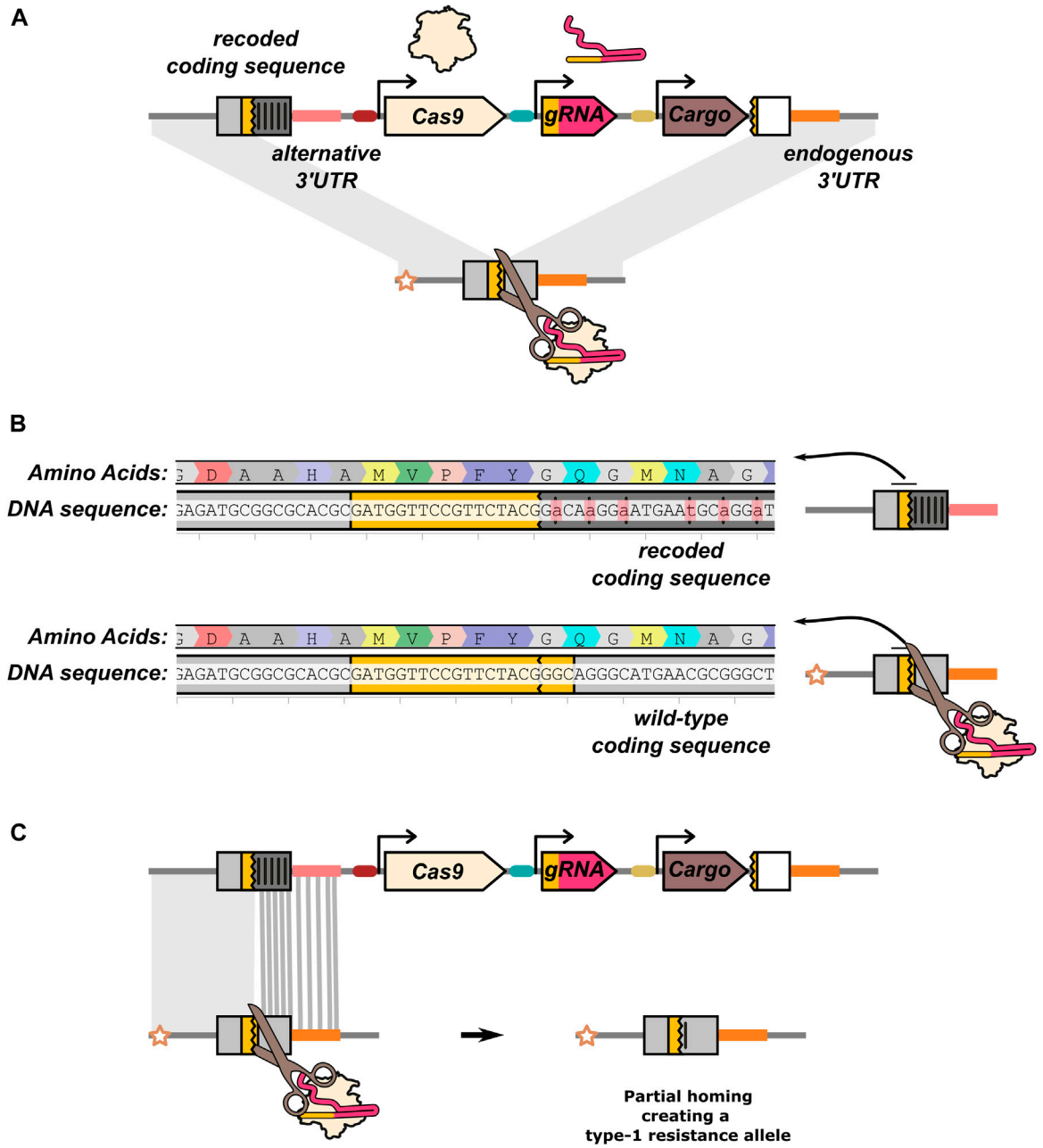

FIGURE 6 | Rescue HEG targeting an essential gene. **(A)** In addition to the nuclease components (Cas9 and gRNA), the drive element carries an effector/cargo gene and a "rescue" sequence that restores the function of the gene the HEG is disrupting. The rescue sequence allows the drive element to be more fit than type-2 resistance alleles. The effector function of the rescue HEG is mediated by a separate cargo gene. In all other illustrations in this manuscript, the effector function of the drive is assumed to be mediated by disruption of the target gene and the HEG does not carry a rescue. **(B)** In this example, the rescue is a recoded version of an endogenous gene. Synonymous codon changes have been made to prevent partial homing (and recognition by the gRNA). **(C)** The recoded gRNA target sequence is located at the start of the rescue sequence. Partial homing by means of any part of the recoded sequence would generate a type-1 resistance allele. The recoding and gRNA target sequence is taken from (Pham et al., 2019).

While efforts have been made to experimentally validate the intended transgene expression pattern (Hammond et al., 2016; Kandul et al., 2020; Terradas et al., 2021c; Weitzel et al., 2021), there are fewer cases where this has been done throughout development (Li et al., 2017). The result of this is that any hypothesis about the ideal expression/activity pattern for HEGs is currently essentially unfalsifiable as any exception to a proposed hypothesis can easily be explained away by the many ways a drive may fail to recapitulate the intended expression pattern, at the needed expression level. Improved methods to test different

activity patterns (e.g., drug inducible Cas9 (López Del Amo et al., 2020b; Chae et al., 2020)) and high-throughput methods to track HEG expression and nuclease activity (e.g., Cas9-based lineage tracing (McKenna et al., 2016)) are sorely needed to validate our assumptions about the underlying factors influencing interchromosomal HDR. This becomes increasingly important as evidence emerges that interchromosomal HDR can occur before the formation of the mature germline (López Del Amo et al., 2020a; Kandul et al., 2020; Filler-Hayut et al., 2021; Li et al., 2021).

Many approaches have been developed to control the expression and activity of transgenes. However, the use of modular systems such as GAL4–UAS to enforce a new HEG activity pattern will likely be more challenging than the current trial-and-error approach of identifying and testing new promoter/5′UTR and 3′UTR regulatory sequences. Yet, we expect that this type of modular approach will enable high-throughput design-build-test cycles. The modularity gained with such an approach will in turn improve our ability to draw conclusions about the underlying biology affecting HEG efficiency and increase the robustness of new designs going forward.

While multiplexing may have diminished returns in improving homing efficiency with standard approaches, it may nonetheless greatly diminish the likelihood of type-1 resistance alleles emerging. The targeting of highly conserved sequences in essential genes has proved beneficial for reducing the impact of resistance alleles. If approved, we expect that the current "state-of-the-art" HEGs in *Anopheles* mosquitoes may progress on to field trails without substantial additional changes to their core design. If this is the case, the complexity of a real-world release will be the ultimate test of the HEG technology. Our constantly expanding genetic "toolbox" and optimisation strategies provide hope that HEGs may be a highly effective tool for combating the harms caused by a broader set of medically and agriculturally relevant (homing refractory) insect pests.

AUTHOR CONTRIBUTIONS

SV wrote the initial draft and JA wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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GLOSSARY

Gene drive: Gene drive refers to both the process by which a genetic element biases its own inheritance, and the genetic element itself (Alphey et al., 2020). Frequently shortened to just "drive" for either meaning.

Homing endonuclease gene (HEG) drive: A gene drive can be composed of multiple distinct genetic elements. A form of gene drive that functions through the cleaving of a specific genomic locus followed by homology-directed repair (HDR) using the homologous chromosome as the template. Inheritance of the drive allele is increased when the nondrive allele is converted to a drive allele in the germline linage of an individual heterozygous for the drive. In the context of this review, different implementations of the core HEG design will be discussed as different drives. HEG-based drives are also commonly referred to as "homing drives".

Nuclease: An enzyme that can catalyse the cleavage of nucleic acids. For HEGs, sequence-specific endonucleases are used.

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats Associated Protein 9. An endonuclease protein that can be programmed to specifically cut a DNA sequence through a guide RNA (gRNA). Cleavage requires an additional three base-pair protospacer adjacent motif ("NGG") specified by interactions of the Cas9 protein with the target DNA.

gRNA: A ≈100 nucleotide RNA that complexes with the Cas9 protein and specifies a ≈20 nucleotide long target sequence to be cut by RNA:DNA base-pairing.

Gene drive allele/element: Genetic components that contain all or a subset of the genetic parts needed to induce gene drive. Gene drive mechanisms rely on endogenous pathways (such as DNA repair), but these are not considered part of the gene drive itself.

Target allele: The target, often wild-type, allele that the HEG can cut and convert. It can also be a "synthetic" target site specifically introduced to contain the HEG's recognition sequence. In research contexts, the target is often a (haplosufficient) gene that, when disrupted, gives a phenotypic readout.

Donor and Recipient chromosome: In a heterozygous individual, the HEG is designed to copy itself from the "donor" chromosome to the "recipient" chromosome. In some studies, the homologous chromosomes can be differentiated by a marker separate from the drive allele. This allows the original donor alleles to be distinguished from the newly created drive alleles on the recipient chromosome.

Homing/Drive conversion rate: Conversion of a target allele to a drive allele through interchromosomal HDR. Commonly measured as the fraction of target alleles that have been converted to a drive allele in the germline lineage of a drive allele heterozygote. If either the target or the drive allele have a tightly linked marker, this can be calculated very precisely. If not, it is estimated from the inheritance of the drive compensated for the 50% expected inheritance from a heterozygote through nondrive Mendelian inheritance.

Type-1 resistance mutations: A functional classification of mutations in the HEG target gene that leave the target gene's function intact. This is target dependent, and the same sequence changes can result in different functional outcomes for different genes and different sites within the same gene.

Type-2 resistance mutations: Mutations in the HEG target that disrupt the gene's normal function.

Unconverted target alleles: Target alleles that have not been mutated or converted by the HEG. It is possible that the unconverted target alleles were cut, but DNA repair did not introduce any mutations. For many crosses, without additional molecular assays, intact target alleles cannot be phenotypically differentiated from type-1 resistance mutations.

Deposition: HEG nuclease protein, mRNA, or gRNA that has been expressed in the parent and carried over to the progeny. Deposition does not require the progeny to have inherited the gene that expressed those HEG components in their parent(s). However, the inheritance of a HEG allele can influence the outcome of deposition.

Shadow drive: Biasing of a particular allele, presumably through homing, that occurs as a result of deposited HEG components.

Somatic phenotype: HEGs are commonly tested by targeting a gene that gives a somatic phenotype when disrupted, such as a yellow body or white eyes in insects. For many experimentally tested HEGs, heterozygous individuals do not present with a somatic phenotype unless nuclease activity has caused the wild-type target allele to be replaced by the drive allele or a type-2 resistance mutation in a substantial proportion of somatic cells.

Knock-out phenotype: Uniform phenotype which is consistent with the target gene having been disrupted in most or all cells of that tissue (e.g., completely white eyes).

Mosaic phenotype: An intermediate phenotype between wild-type and the knock-out phenotype consistent with some cells within the organism retaining function of the target gene and the target gene having been disrupted in other cells.

Somatic nuclease/drive activity: Activity of the HEG nuclease in somatic tissues. In almost all cases this is not intended.

Germline restricted nuclease/drive activity: Activity of the HEG nuclease in cells of the germline. Characterised by changes to the gamete frequencies without generating a somatic phenotype.

Embryonic nuclease/drive activity: Activity of the drive in the early embryo. Often characterised by both (error-prone) germline and somatic drive activity. Associated with individuals that have experienced parental deposition.

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Genetic Stability and Fitness of *Aedes aegypti* Red-Eye Genetic Sexing Strains With Pakistani Genomic Background for Sterile Insect Technique Applications

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The mosquito species *Aedes aegypti* is the primary transmitter of viruses that cause endemic diseases like dengue in Pakistan. It is also a cause of other vector-borne diseases like yellow fever, Zika fever, and chikungunya, which significantly impact human health worldwide. In the absence of efficient vaccines (except for yellow fever) or drugs, vector control methods, such as the sterile insect technique (SIT), have been proposed as additional tools for the management of these diseases. Mosquito SIT programs are based on the release of sterile males and it is important female releases to be ideally zero or to be kept at a minimum, since females are the ones that bite, blood-feed and transmit pathogens. Recently, an *Ae. aegypti* genetic sexing strain (GSS), with and without a recombination-suppressing inversion (Inv35), was developed using the eye color as a selectable marker, with males having black eyes and females red eyes. In the present study, we introgressed the sexing features and the Inv35 of the *Ae. aegypti* red-eye GSS into the Pakistani genomic background aiming to their future use for SIT applications in the country. Both introgressed strains, the Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK, were evaluated in respect to their genetic stability and biological quality by assessing parameters like recombination rate, fecundity, fertility, pupal and adult recovery, time of development, pupal weight, survival, and flight ability in comparison with a wild Pakistani population (PAK). The results suggest that the sexing features and the recombination suppression properties of Inv35 were not affected after their introgression into the local genomic background; however, some biological traits of the two newly constructed strains were affected, positively or negatively, suggesting that a thorough quality control analysis should be performed after the introgression of a GSS into a new genomic background prior to its use in SIT field trials or applications. The importance of using GSS with local genomic background for SIT applications against *Aedes aegypti* is also discussed.

Keywords: area-wide integrated pest management, vector control, dengue, Zika, insect pest control

INTRODUCTION

Aedes aegypti mosquitoes are responsible for the transmission of numerous viral infections among humans (Bhatt et al., 2013) in particular considered as a major vector of viruses that are responsible for diseases like dengue, chikungunya, Zika fever, and yellow fever (Morrison et al., 2008; Souza-Neto et al., 2019). It has spread over the world's tropical and subtropical regions and breeds in artificial containers within human environments to have easy access to blood for feeding and almost no predators (Brown et al., 2011).

Dengue, along with the other infections mentioned above, is becoming a global public health concern due to its rapid geographical spread in parallel (Guzman and Harris, 2015; Kraemer et al., 2015). In Pakistan, dengue has also become more common over recent decades and has been spreading at an alarmingly high rate, with cases being reported from urban and rural areas from different regions of the country (Khan et al., 2018). Numerous factors like climatic changes, public unawareness, inadequate surveillance, and insufficient funding have contributed to frequent dengue outbreaks (Ahmad et al., 2017).

In addition to the reduction of dengue transmission, vector control is also desirable to avoid nuisance and hypersensitivity/allergies mediated by bites (Paris et al., 2011; Bowman et al., 2016; Barrera et al., 2017). Presently, vector control mainly depends on insecticides applied on mosquito larval habitats and against adult mosquitoes indoors and during dengue outbreaks. However, the selective pressure on populations resulting in insecticide resistance has become an issue for chemical control in several *Ae. aegypti* mosquito populations worldwide. Furthermore, only a few new insecticides have been commercialized for dengue vector control (Vontas et al., 2012; Smith, 2016). In many Pakistani field populations of dengue vectors, it is common to find insecticide resistance at moderate to high levels, which has been already reported as a leading future problem regarding vector control (Khan et al., 2011; Arslan et al., 2016). Particularly in urban areas, *Ae. aegypti* has been reported to develop resistance against commonly used insecticides (Jahan and Shahid, 2013).

As conventional control methods are not effective enough, environmentally friendly and species-specific approaches such as the sterile insect technique (SIT) are needed to control mosquito vector populations (Bouyer and Lefrançois, 2014; Carvalho et al., 2014; Lees et al., 2015; Bourtzis et al., 2016). SIT is an insect pest control method which is based on the release of sterile males to suppress, prevent the (re)introduction, contain or even locally eradicate insect pest populations. SIT has been in use for decades as an effective tool to suppress or even eliminate numerous insect pests such as the New World screwworm, tsetse fly, Mediterranean fruit fly etc. (Baumhover, 1966; Ciss et al., 2019; Gutierrez et al., 2019; Dyck et al., 2021b).

SIT is a species-specific and environmentally friendly method to control populations of insect pests and disease vectors (Dyck et al., 2021a). In SIT, radiation sterilizes male mosquitoes, which are released in the open environment to mate with wild females, thus resulting in reduced fertile crosses and subsequent population suppression (Dyck et al., 2021a). A successful SIT mosquito release program's primary obstacle is eliminating or separating the females because, in this case, only females bite and transmit the etiological agent. Therefore, removing females prior to sterile males' release is a strict prerequisite (Gilles et al., 2014; Papathanos et al., 2018; Lutrat et al., 2019).

Sex separation strategies currently available are time- and labor-consuming, and highly prone to errors. Efficient and robust sex separation methods are not yet fully available in mass-rearing facilities (Gilles et al., 2014; Papathanos et al., 2018; Zacarés et al., 2018; Lutrat et al., 2019; Zheng et al., 2019; Crawford et al., 2020). In addition, genetic and molecular-based approaches can be exploited for the development of more convenient, reliable, efficient and cost-effective methods for mosquito sex separation at a mass-rearing scale (Gilles et al., 2014; Papathanos et al., 2018; Lutrat et al., 2019). For example, genetic sexing strains (GSS) with phenotypic markers to distinguish male from female mosquitoes may prove useful. An excellent example of GSS developed and reared in mass rearing facilities worldwide for SIT purposes are the VIENNA 7 and VIENNA 8 GSS of the Mediterranean fruit fly *Ceratitis capitata*, which are based on a color and a thermal lethality mutation linked to the sex (Augustinos et al., 2017; Franz et al., 2021).

Recently, such a GSS for *Ae. aegypti* was developed through classical genetics by exploiting the red-eye mutation (*re*) as a phenotypical marker, resulting in females with red eyes and males with black eyes through all the developmental stages (Koskinioti et al., 2021). However, this strain still had recombinants, which would compromise the genetic stability and the GSS efficiency. A radiation-induced chromosomal inversion (Inv35) was then introduced as a recombination suppressor to enhance its genetic stability (Augustinos et al., 2020). Through laboratory-scale quality control tests, it was evident that the strain exhibited sufficient biological quality to be considered as a candidate for *Ae. aegypti* SIT programs (Koskinioti et al., 2021). In a subsequent study it was shown that the recombination frequency in the GSS strains, with and without the inversion, is not affected if the red-eye mutation and the Inv35 are introduced to six different genomic backgrounds, Brazil, Indonesia, Mexico, Sri Lanka, Singapore, and Thailand (Augustinos et al., 2022).

However, it is known that the background genotype contributes significantly to the biological quality and the performance of insect strains aimed for releases especially in terms of mating success (Quintero-Fong et al., 2016; Carvalho et al., 2020; Leftwich et al., 2021). Laboratory reared insects differ from wild ones due to combined effects of bottlenecking, high inbreeding, selection for artificial rearing, and genetic variation. In the present study, we introgressed the red-eye mutation and the chromosomal inversion Inv35 into the local (Pakistani) genomic background and the two new GSS developed, Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK were evaluated in respect to their genetic stability, biological quality, and their potential to be used for SIT applications against *Ae. aegypti* populations in Pakistan.

MATERIALS AND METHODS

Aedes aegypti Strains and Rearing Conditions

The Rexville red eye mutant strain used in the present study is a long-domesticated laboratory strain (kindly provided by Dr. Margareth Capurro at the Department of Parasitology, University of Sao Paulo, Brazil) and had previously been used in other studies including the construction of the *Ae. aegypti* red-eye GSS (Costa-da-Silva et al., 2017; Augustinos et al., 2020, 2022). The color of both compound and simple eye of this strain remains red throughout development. The chromosomal inversion (Inv35) was induced through irradiation, and it is known to suppress recombination between the red eye and M loci (Augustinos et al., 2020; Koskinioti et al., 2021). PAK is a recently domesticated strain from mosquitoes collected from Northern areas of Pakistan's KP Province and was used as a source of the local genomic background. The introgression of the red eye mutation and Inv35 was performed as described previously (Augustinos et al., 2022), consisted of a series of backcrosses and was expected to result into two new GSS, Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK with ~98.8 and ~98% PAK genetic background, respectively. The two new GSS were evaluated for their genetic stability until the seventh generation while their biological quality was assessed at the third generation in the present study.

All strains were kept under standard rearing conditions (FAO-IAEA, 2017). More specifically, mosquitoes were maintained in the insectary of the Insect Pest Control Laboratory of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria, at 27 ± 1°C, 80% RH, and a photoperiod of 12/12 h day/night, including 1 h of twilight. Females of all strains were blood-fed for 20 min, two times per day for two consecutive days per week, using collagen casing with porcine blood. In addition, 10% sugar solution was provided continuously in 30 × 30 × 30 cm adult plastic cages (BugDorm-1, MegaView Science Co., Taiwan). Eggs were collected by keeping moistened white filter paper in urine cups half-filled with water at least 72 h after blood feeding.

Genetic Stability

In each generation, after sorting pupae in glass pupal sorter, a minimum of 1,000 male and female pupae were screened under a common stereomicroscope. Both expected and recombinant genotypes were counted separately in male and female pupae by observing the genital lobe and the eye color. Data was recorded in the appropriate spreadsheet for each generation. For strains maintained under filtering, males with red eyes and females with black eyes (recombinants) were counted and removed from the colonies (Koskinioti et al., 2021). For non-filtered colonies, and following counting recombinants, all insects were transferred in the same cage to set up the next generation.

Biological Quality

Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK, both at the third generation, were compared with a recently domesticated PAK strain in respect to the following biological quality parameters:

Fecundity

For each strain, 50 newly emerged males and an equal number of females were released together in a plastic rearing cage (BugDorm-1 rearing cage 30 × 30 × 30 cm) and mated for 3–4 days. Pre-mated females were blood-fed twice per day for 20 min for two consecutive days to ensure full engorgement. Three replicates of 10 fully fed females per small cage (BugDorm-4S1515 with 15 × 15 × 15 cm) were performed for each strain. Eggs were collected for the first two gonotrophic cycles. Dead females (if any) were replaced by other gravid females of the same age. Eggs were counted under a common stereomicroscope before drying. The total number of eggs was divided by 10 to estimate the average number of eggs per female per replicate.

Fertility

Eggs from the fecundity test of each replicate from both gonotrophic cycles were hatched by placing egg papers in airtight glass jars (500 ml), prepared in advance to have water with low dissolved oxygen content (boiled water), and 2–4 drops of larval diet were added to stimulate egg hatching. Jars prepared in the morning were kept in an incubator for hatching at 27°C until the next day (around 24 h). First instar larvae (L1) were counted by aspirating with a 200 µl tip on the plastic pipette. The percentage of hatching was recorded of all three strains.

Recovery Rates and Development Time

Pupal recovery and adult recovery were recorded by counting the total number of pupae and adults respectively, deriving from the total number of eggs. Development time was recorded by counting the number of pupae of each sex collected for each strain every 24 h. The duration of development was estimated from egg hatching to pupation.

Pupal Weight

Ten female and 10 male pupae per replicate were slightly air-dried for 20 min by placing them on a towel paper, observing and shaking the trays until they are not clustered. Batches of 10 pupae each were weighed to calculate the average pupal weight. In total, five replicates were counted for each strain per sex.

Survival Rate

Fifty newly emerged males and females per replicate per strain were kept in small cages BugDorm-4S1515 (15 × 15 × 15 cm). Each cage was provided with 10% sucrose solution. Dead mosquitoes were counted and removed daily. At the end of a 33-day period, dead mosquitoes were counted and subtracted from the total number of adults released to estimate the average survival rate. Three replicates were made per sex per strain.

Flight Ability

Approximately one hundred 4–5 days old adult males of each strain per replicate were tested in a Flight Test Device (FTD) as described previously (Culbert et al., 2018). Three replicates per

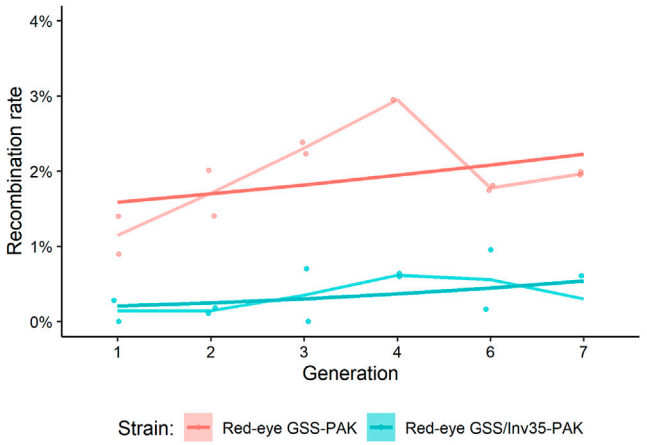

FIGURE 1 | Recombination rate in Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK strains during seven generations. Lighter lines represent the mean of the points, and the darker straight lines represent the GLM line for each strain.

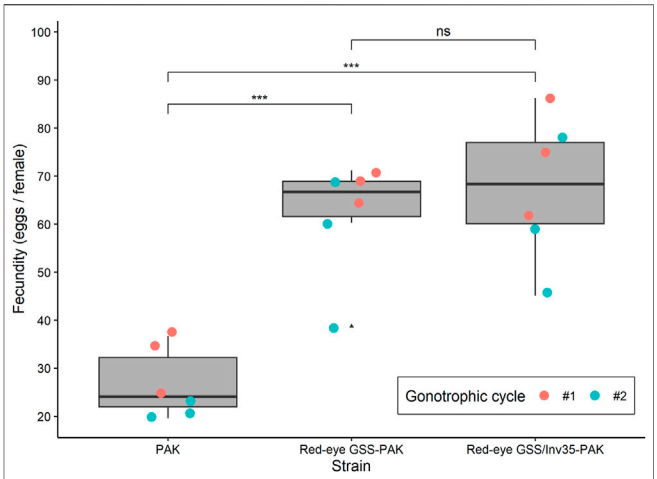

FIGURE 2 | Fecundity of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains during the first and second gonotrophic cycle. Significance symbols: "***" for $p < 0.001$, and "ns" for "not significant".

strain were performed. After 2 h from the release time, successful fliers were aspirated from the outer part of the FTD and were counted. Similarly, unsuccessful fliers trapped in the glass tubes and the releasing arena were also counted. The number of successful fliers out of the total number of adult males released corresponded to the flight ability percentage.

Statistical Analysis

All statistical analysis was performed using R language 4.1.2—"Bird Hippie" (R Core Team, 2020) with RStudio environment—version 2021.09.02 + 382 (RStudio Team, 2016). Normality was assessed by the data frequency distribution and its point distribution of the quantile-quantile plot, and it was then determined whether parametric or not. The alpha < 0.05 was considered statistically significant for all generalized linear models used for each parameter evaluated with a multiple comparison of the mean using Tukey contrasts as post hoc. The model has considered binomial distribution for percentages Poisson distributions for counting, considering logit and log their respective transformations. The box and whiskers plot were used to demonstrate the full data distribution representing the minimum, maximum, median, 1st and 3rd quartiles. Parametric statistical comparisons were only performed using multiple comparisons of means by computing the contrast matrices of all comparisons obtained by each generalized linear model. For survival analysis, the Kaplan-Meier, Log-rank test, and the Cox proportional-hazards model were used to distinguish differences and also to obtain the survival curve plot using the *survival* package (Therneau, 2020). Information about additional packages used in the present study can be found in the supporting material together with all statistical analysis (**Supplementary Material S1**) and the original data used for all analysis (**Supplementary Material S2**).

RESULTS

Genetic Stability

The genetic stability was assessed by recording the expected and recombinant genotypes in the Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK strains up to the seventh generation. In total, 10,211 Red-eye GSS-PAK and 8,479 Red-eye GSS/Inv35-PAK individuals were screened and the recombination rate ranged between 1.15–3.70% and 0.14–0.62%, respectively (**Supplementary Material S2**). The results presented in **Figure 1** confirm that the presence of Inv35 significantly suppresses the recombination rate (Df = 1, F = 25.73, $p = 2.6^{-6}$) (**Figure 1**).

Biological Quality

Fecundity

The introduction of the red eye mutation and the inversion Inv35 into the Pakistani genomic background had a positive impact on the fecundity, which was significantly increased in the first and the second gonotrophic cycle in both GSS. The average fecundity of the PAK strain was 31.97 and 21.60 eggs/female in the first and the second gonotrophic cycle, respectively. On the contrary, the average fecundity of the Red-eye GSS-PAK was 68.60 and 55.67 eggs/female while that of the Red-eye GSS/Inv35-PAK was 74.27 and 60.80 eggs per female in the first and the second gonotrophic cycle, respectively, significantly higher than the values recorded for the PAK strain (Df = 2, F = 26.862, $p = 0.00257$—**Figure 2**). There was no statistically significant difference in the fecundity between the Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK strains (z = −1.94 $p = 0.127$), while the fecundity of both was higher than that of the PAK strain (z = 8.724 and 7.216 with $p = < 1^{-4}$, for Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK respectively).

Fertility

Significant differences were observed among the three strains (PAK, Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK) in respect to the

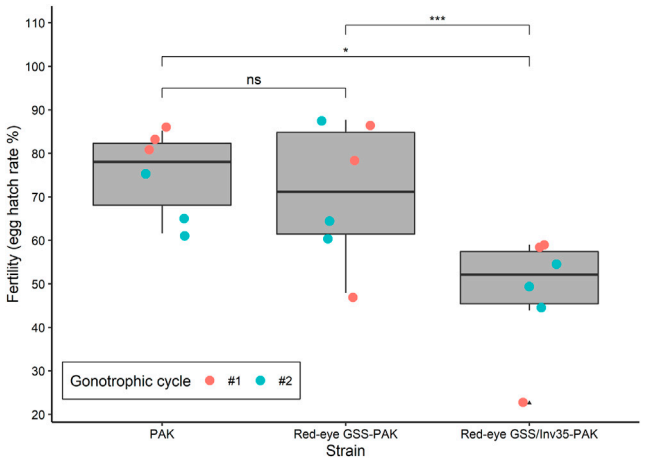

FIGURE 3 | Fertility of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains. Significance symbols: "***" for $p < 0.001$, "*" for $p < 0.05$, "ns" for "not significant".

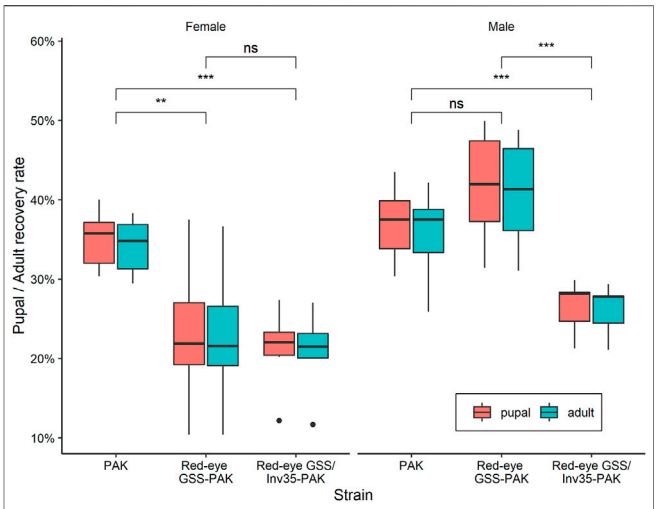

FIGURE 5 | Pupal and adult recovery rate of males and females of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains. Significance symbols: "***" for $p < 0.001$, and "ns" for "not significant".

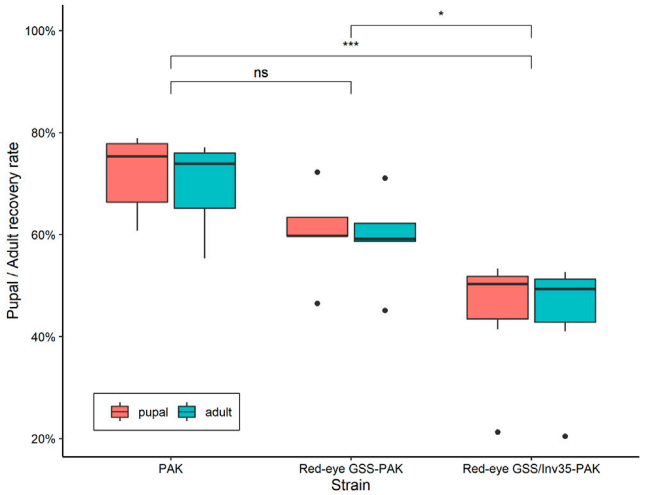

FIGURE 4 | Pupal and adult recovery rate of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains. Significance symbols: "***" for $p < 0.001$, "*" for $p < 0.05$, and "ns" for "not significant".

fertility (egg hatching) (Df = 2, F = 6.062, $p = 0.00799$—**Figure 3**). The average egg hatching of the PAK strain was 75.24%, slightly reduced in Red-eye GSS-PAK to 70.92% with no statistic difference (z = −0.37, $p = 0.92584$), while more pronounced reduction was observed in the Red-eye GSS/Inv35-PAK 48.04% (PAK: z = −2.38 $p = 0.04367$, and Red-eye GSS-PAK: z = −3.12, $p = 0.00487$).

Pupal and Adult Recovery Rates

Statistically significant difference was observed among the strains in respect to the pupal and adult recovery rates. The average pupal and adult recovery were 72 and 70% in the wild type PAK strain, 60 and 59% in the Red-eye GSS-PAK strain, and 45 and 44% in the Red-eye GSS/Inv35-PAK strain, respectively (Pupal: Df = 2, F = 11.18, $p = 0.00125$; Adult: Df = 2, F = 9.434, $p = 0.00254$—**Figure 4**).

There was no statistically significant difference between the PAK and Red-eye GSS-PAK strains in respect to these two traits (z = −1.98 (pupal), $p = 0.1167$ and z = −1.72 (adult), $p = 0.199$). However, significant reduction was observed in both pupal and adult recovery rates between the Red-eye GSS/Inv35 and the other two strains (PAK: z = −4.56 (pupal) and −4.18 (adult), both with $p = 0.001$, Red-eye GSS-PAK: z = −2.46 (pupal), $p = 0.0367$ and −2.34 (adult), $p = 0.05$). Statistically significant differences were also observed among the three strains in respect to the pupal and adult recovery rates of males and females of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains (Df = 1, F = 136.72, $p = 8.83^{-7}$—**Figure 5**).

The average female and male pupal recovery rate were 35 and 37% for PAK, 23 and 42% for Red-eyes GSS-PAK, and 21 and 26% for Red-eyes GSS/Inv35-PAK, respectively. On the other hand, the average female and male adult recovery rate were 34 and 36% for PAK, 23 and 41% for Red-eyes GSS-PAK, and 21 and 26% for Red-eyes GSS/Inv35-PAK. Statistical analysis indicated significant difference in female pupal and adult recovery rates between PAK and Red-eye GSS-PAK (pupal: z = −2.98, $p = 0.00787$, and adult: z = −2.91, $p = 0.00989$), and between PAK and Red-eyes GSS/Inv35-PAK strain (pupal: z = −3.48, $p = 0.00146$, and adult: z = −3.44, $p = 0.00166$), but not between Red-eye GSS-PAK and Red-eyes GSS/Inv35-PAK (pupal: z = −0.55, $p = 0.84643$, and adult: z = −0.524, $p = 0.85959$) (**Figure 5**) In respect to the male pupal and adult recovery rates, the statistical analysis indicated no differences between PAK and Red-eyes GSS-PAK (pupal: z = 1.46, $p = 0.30929$, and adult: z = 1.54, $p = 0.2745$); however, there was difference between PAK and Red-eyes GSS/Inv35-PAK (pupal: z = −3.27, $p = 0.00315$, and adult: z = −2.86,

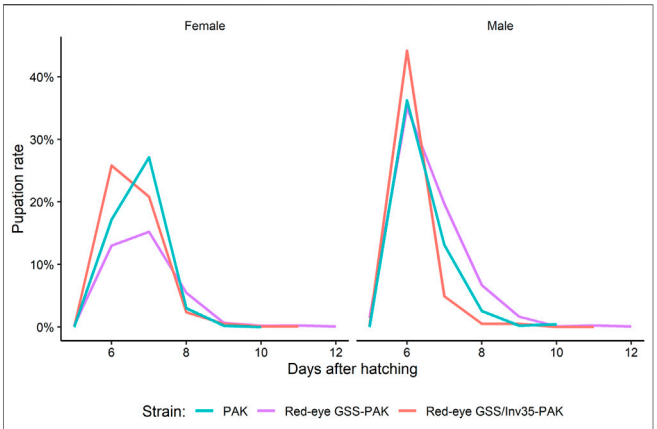

FIGURE 6 | Pupation curve of males and females of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains.

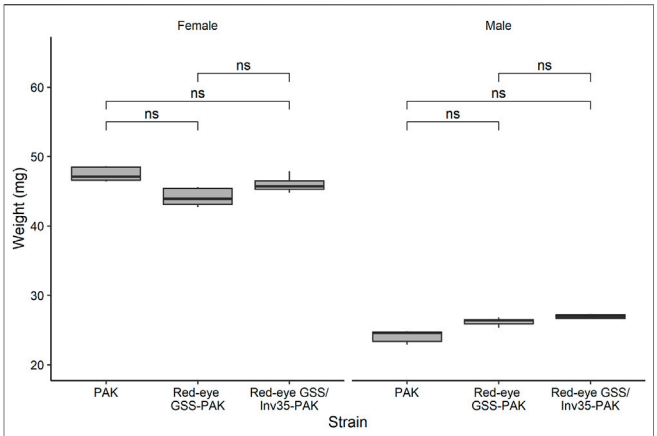

FIGURE 7 | Pupal weight of males and females of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains. Significance symbol: "ns"—not significative.

p = 0.0119) as well as between Red-eyes GSS-PAK and Red-eyes GSS/Inv35-PAK (pupa: z = −4.62, p = 0.001, and adult: z = −4.28, p = 0.001) (**Figure 5**).

There was no significant difference among the three strains on the pupation rate of males and females (Df = 1, F = 0.314, p = 0.57951). The maximum pupation rate for males was on the sixth day for all three strains. For females, the peak was observed on the seventh day for PAK and Red-eye GSS-PAK and on the sixth day for Red-eye GSS/Inv35-PAK (**Figure 6**).

Pupal Weight

No statistically significant differences were observed among the strains in respect to the weight (Df = 2, F = 1.776, p = 0.962) but, as expected, female pupae were heavier than male ones in all three strains studied (Df = 1, F = 1,194, $p = 2^{-16}$—**Figure 7**).

Survival Rate

Male survival rate of the Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK strains was significantly reduced compared to PAK (Likelihood ratio test = 24.36, df = 2, $P = 5^{-6}$—**Figure 8A**) while no statistically significant difference was observed in female survival rate (Likelihood ratio test = 0.7, df = 2, p = 0.7—**Figure 8B**) during the first 30 days period post emergence. It should be noted, however, that more than 85% of Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK males were alive after the end of the observation period.

Flight Ability

In respect to the flight ability, the statistical analysis presented significant differences among the three strains (Df = 2, F = 11.74, p = 0.00844—**Figure 9**) with mean percentage of successful flyers being 65, 73 and 82% for the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-Pak strains. There was no difference between the PAK and Red-eye GSS-PAK strains (z = 2.04, p = 0.1016); however, there was significant difference between PAK and Red-eye GSS/Inv35-Pak (z = 4.78, p = 0.001) as well as between Red-eye GSS-PAK and Red-eye GSS/Inv35-Pak strains (z = 2.87, p = 0.0115).

DISCUSSION

Efficient, cost-effective, and safe SIT applications against major vector mosquito species, such as *Ae. aegypti* and *Ae. albopictus*, depend on efficient sex separation methods and the release of sterile males. The removal of *Aedes* female mosquitoes is needed because they bite, blood-feed and transmit pathogens such as chikungunya, dengue, yellow fever and ZIKA (Gilles et al., 2014; Papathanos et al., 2018; Lutrat et al., 2019). Current small-scale SIT trials are being carried out using local populations to minimize the risks associated with the introduction of vector mosquitoes of different origin (Bouyer et al., 2020; Carvalho et al., 2020; WHO and IAEA, 2020). Indeed, releasing GSS males carrying the local genomic background will enhance the efficiency of SIT since their mating competitiveness is likely to be higher than that of males of different origin. In addition, the SIT application with local males is not expected to raise biosafety and biosecurity concerns compared to a trial which would be based on mosquitoes originated from a different geographical region (Bouyer et al., 2020; WHO and IAEA, 2020; Augustinos et al., 2022). In the present study, the red eyes mutation and the inversion Inv35, which were used in the initial construction of the *Ae. aegypti* Red-eye GSS and Red-eye GSS/Inv35 strains (Augustinos et al., 2020; Koskinioti et al., 2021), were introduced into the genomic background of a wild-type PAK strain to assess their impact on their genetic stability, biological quality, and potential for SIT applications.

The genetic stability of GSS highly depends on recombination phenomena, which usually occur in males (Franz et al., 2021). Filtering systems and chromosomal inversions have been proposed as tools for the suppression of recombination and/or the removal of recombinants in order to maintain the genetic integrity of GSS (Franz et al., 2021). Unlike in fruit flies, genetic recombination occurs in both males and females of *Aedes* species, and this can

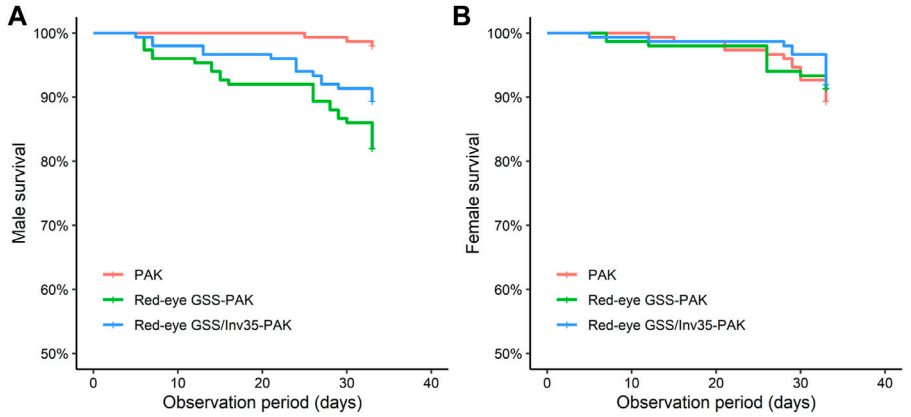

FIGURE 8 | Survival rate of males **(A)** and females **(B)** of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains during the first 33 days post-emergence observation period.

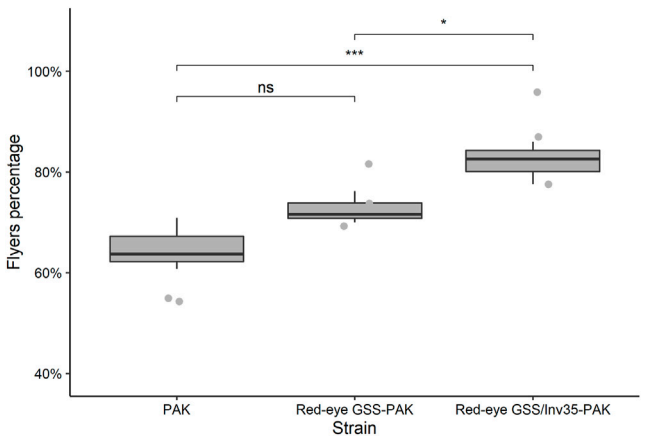

FIGURE 9 | Flight ability of males of the PAK, Red-eye GSS-PAK, and Red-eye GSS/Inv35-PAK strains. Significance symbols: "***" for $p < 0.001$, "*" for $p < 0.05$, and "ns" for "not significant".

significantly affect their stability, especially in the context of preserving the colony's genetic integrity under mass rearing and female contamination in male-only releases for SIT programs (Augustinos et al., 2020; Franz et al., 2021). In our recently published studies, we reported the construction of the red-eye GSS (Koskinioti et al., 2021) and the introduction of Inv35 to suppress recombination (Augustinos et al., 2020). However, the recombination rate as well as the overall performance of a strain depends on several factors, including genomic background (Ouda and Wood, 1985; Augustinos et al., 2020, 2022; Carvalho et al., 2020). Therefore, in the present study, we introgressed the red eye mutation and the chromosomal inversion Inv35 in a wild population from Pakistan to assess the impact of the local genomic background on the genetic stability and the biological quality of newly constructed strains under laboratory rearing conditions.

As concerns the genetic stability, our results showed that genetic recombination was significantly suppressed in the presence of Inv35 and that the overall recombination rate in the Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK strains was in the same range as described in the original strains, Red-eye GSS and Red-eye GSS/Inv35, reported in our previous study (Koskinioti et al., 2021). Taken together these data suggest that the genomic background did not have a significant impact on the genetic stability of the genetic sexing strains and are in accordance with recently reported recombination-suppressing properties of Inv35 (Augustinos et al., 2022).

One of the most important requirements for a successful SIT mosquito program is to mass produce and release high-quality sterile males that can compete with wild males for mating wild females (Bouyer and Vreysen, 2020; Parker et al., 2021). Quality of males is essential to determine the number of males to be released in the field, and high productivity, proper mating behavior, high survival, and good flight ability are among the desirable characters (Culbert et al., 2018; Parker et al., 2021). In the present study, we also determined the impact of the local Pakistani genomic background on the biological quality of the Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK under laboratory conditions by assessing parameters like fecundity, fertility, pupa and adult recovery, time of development, pupal weight, survival, and flight ability in comparison with the wild-type PAK strain.

Our results showed that the introgression had a positive impact on the fecundity of Red-eye GSS-PAK and Red-eye GSS/Inv35-PAK strains in both the first and the second gonotrophic cycle, similar to that reported for the originally constructed Red-eye GSS (Koskinioti et al., 2021). However, female pupal and adult recovery rate, and male survival rate were negatively affected. A positive impact on the flight ability of Red-eye GSS/Inv35-PAK males, compared to both PAK and Red-eye GSS-PAK males, was observed which could be attributed to heterozygote advantage. It is also important to note that the fertility as well as the male pupal and adult

recovery rate was reduced in the Red-eye GSS/Inv35-PAK strain. On the other hand, the introgression had no effect on the pupation rate of males and females, and the pupal weight. The latter observation is very important in case a novel sex separation approach is developed based on both selectable markers, pupal size and eye color, as recently suggested (Koskinioti et al., 2021). In addition, it should be noted that, although the flight ability is a good indicator for the biological quality of males, proper evaluation of the male mating competitiveness of the Red-eye GSS and Red-eye GSS/Inv35 will be required prior to their use in any small- or large-scale field applications.

CONCLUSION

Although the actual performance of a potential SIT strain can only be assessed in open-field conditions, laboratory characterization regarding genetic stability and biological quality is of utmost importance prior to mass production and releases of sterile males. Our present study studied biological traits or parameters, such as genetic recombination, fecundity, fertility, pupa and adult recovery, time of development, pupal weight, survival, and flight ability of two newly constructed introgressed strains Red-eye GSS-PAK and the Red-eye GSS/Inv35-PAK in comparison to the wild-type PAK strain. The results indicated that important biological quality parameters such as fecundity, fertility, pupal and adult recovery rate, survival rate, and flight ability, can be affected during the introgression process of different factors, such as the red-eye mutation and Inv35, into a new genomic background, which is in agreement with previous reports (Carvalho et al., 2020). Interestingly, some of these traits were affected in a sex specific manner. It is therefore recommended that the transfer of the selectable marker (red eye) and/or chromosomal inversion (Inv35) of the *Ae. aegypti* red eye GSS into new genomic backgrounds for the construction of the respective GSS should be accompanied by a thorough evaluation of the genetic stability and biological quality prior to its use in SIT applications in the field.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Conceptualization, KB and AA; methodology, KB and AA; validation, formal analysis, DC; investigation, and data curation, MM-U-H, DC and LD; writing original draft preparation, MM-U-H; writing review and editing, KB and AA; supervision, KB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.871703/full#supplementary-material

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ORIGINAL RESEARCH
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Effects of Chilling and Anoxia on the Irradiation Dose-Response in Adult *Aedes* Mosquitoes

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The success of the sterile insect technique (SIT) relies on the achievement of high levels of sterility and mating success of the factory-reared sterile males and thus their biological quality, which can be enhanced by the reduction of stress factors encountered during rearing, handling, and irradiation procedures. The achievement of consistent sterility levels requires reliable and standard irradiation protocols. Additionally, mosquito adults require immobilization prior to, and during irradiation to increase processing efficiency and to avoid physical damage caused by movement in restricted space. Common methods for immobilization include chilling and anesthetics such as nitrogen. Here we assessed the effects of chilling and exposure to nitrogen on the irradiation dose-response of *Aedes* mosquitoes, and their downstream effects on some male quality parameters including longevity and flight ability. We found that chilling does not incur damage in the insects in terms of longevity and flight ability when chilling duration and temperature are carefully controlled, and a recovery phase is provided. Irradiation in nitrogen shows high radioprotective effects during irradiation, resulting in reduced induction of sterility. Overall, longevity of males can be improved by irradiating in anoxia, however the exposure to nitrogen itself comes with negative impacts on flight ability. The results reported here will assist in the standardization and optimization of irradiation protocols for the SIT to control mosquito populations of medical relevance.

Keywords: induced sterility, mosquito, irradiation, *Aedes aegypti*, *Aedes albopictus*

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1 INTRODUCTION

The sterile insect technique (SIT) (Dyck et al., 2021) is a biological insect population control tactic that reduces the dependence of insecticides and thus agrees with present day concerns regarding human health and the environment. The SIT concept has been in existance since the 1930's, and has been implemented against various crop pests with huge success since the 1950's.

Abbreviations: AW-IPM, Area-Wide Integrated Pest Management; DO, dissolved oxygen; FAO, Food and Agriculture Organisation; IAEA, International Atomic Energy Agency; IPCL, Insect Pest Control Laboratory; IS, induced sterility; SIT, sterile insect technique; VAS, ventral air space.

It was first implemented against mosquitoes in the 1960's with varying results (Dame et al., 2009), however, the technique has more recently regained interest in the fight against malaria, and in response to the Zika virus outbreaks in 2015. Following increasing demands from Member States, the Insect Pest Control Laboratory of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture has been developing the SIT package for select disease transmitting mosquito species, in particular *Aedes aegypti* and *Ae. albopictus* (the main vectors of dengue, Zika, chikungunya among other arboviruses) and *Anopheles arabiensis*, an important vector of malaria. Great progress has been made for each component of the SIT, resulting in the development of equipment, methods and guidelines for mass rearing, sex separation, irradiation, packing, transportation, quality control, release methods, and field trials. The most notable achievements of the past decade are reviewed in (Vreysen et al., 2021).

The success of the SIT relies on the reliable induction of sterility in the target insect population by releasing mass produced sterile males into the field, where they must outcompete wild counterparts to secure mating which results in no offspring. For this, dependable irradiation protocols are required to ensure constant, and high levels of induced sterility, whilst maintaining the highest possible quality in the sterile insects. The irradiation of medically important mosquito species in the frame of the SIT requires the males to be near to fully sterile both to avoid a risk of replacement of the target population (WHO and IAEA, 2020), and to ensure a maximal efficiency of the sterile males given the high reproduction rate of these species (Aronna and Dumont, 2020). This can be achieved by exposing pupae or adults to ionizing radiation- usually in gamma-ray irradiators (Helinski et al., 2009 and references within), and more recently, in X-ray irradiators (Mastrangelo et al., 2010; Yamada et al., 2014; Du et al., 2019; Zheng et al., 2019), and possibly with industrial accelerators producing electron beams (Balestrino et al., 2016), although these devices are currently used mainly in phytosanitary applications (Smittle et al., 1991; Dohino et al., 1997; Todoriki et al., 2006; Koo et al., 2012).

Although there is a high degree of reliability when achieving expected sterility levels by exposure to a known dose according to dose-response studies, some physical factors influence the dose response in mosquitoes, and biological factors also affect their general sensitivity to radiation. Some of these factors have been studied more frequently, such as the effects of life stage, gender and pupal age (Wakid et al., 1976; Helinski et al., 2006, 2009; Balestrino et al., 2010; Akter and Khan, 2014) wheras very few, or only very old reports exist for the evaluation of others factors, such as effects of hypoxia or anoxia, temperature, and dose rate during radiation exposure (Hallinan and Rai, 1973; Curtis, 1976; El-Gazzar et al., 1983; Ernawan et al., 2017; Zhang et al., 2020).

More recently, a series of experiments to assess the impacts of several biological and physical factors (e.g., strain geographical origin, pupal age pupal size, atmospheric conditions) on dose-reponse in mosquitoes were conducted (Yamada et al., 2019; Yamada et al., 2020) with the aim to develop standardized protocols for the irradiation of mosquito pupae (FAO/IAEA, 2019). However; standardizing irradiation protocols for pupae is difficult, especially in practical terms in large-scale SIT programmes for the following reasons: Pupal age is an important factor that significantly impacts dose-response (Balestrino et al., 2010; Yamada et al., 2019). Although guidelines exist for the optimization of larval rearing for synchronized pupae development (FAO/IAEA, 2020), it is in reality unrealistic to narrow the pupation window to 16 h or shorter, to ensure that all pupae are aged 30 h or older during the irradiation process. Also, timing the pupation so that the collection, sexing and irradiation can occur during daytime working hours is another challenge. Irradiating mixed age batches is not recommended, as irradiating younger pupae can negatively affect adult quality (Balestrino et al., 2010), and over-dosing (as younger pupae require less dose) would further exacerbate this. Conversely, irradiating younger pupae at an optimal dose (to achieve >99% sterility) is possible, however the risk remains that older pupae would be under-dosed, leading to potentially releasing sub-sterile males, in addition to males with diminished quality, thereby compromising success of the otherwise effective SIT. Additionally, and equally problematic is that it is difficult, if not impossible to control the atmospheric conditions surrounding pupae during irradiation in bulk. For mass irradiation at the pupal stage, the pupae would need to be placed in sufficient water within the irradiation canister to provide buoyancy to avoid the pupae at the bottom being crushed. However, this creates a hypoxic environment as pupae submerged in water continue to respirate through their cuticle and quickly deplete the surrounding water of dissolved oxygen (Yamada et al., 2020). As hypoxia reduces irradiation effects, the irradiation of pupae in water results in differential levels of sterility within the sample (Yamada et al., 2020), therefore this method for irradiation cannot be reliable unless, again, the full cohort is significantly overdosed. Apart from quality costs of over-dosing, pupae exposed to hypoxia suffer additional stress and loss in quality. Large numbers of pupae can also be irradiated without water in monolayers, however, pupae are still closely packed and pockets of hypoxia still occur within the sample (Louis Clement Gouagna personal communication) resulting in a proportion of pupae maintaining unacceptable levels of fertility. Drying pupae and spreading them in a manner that would avoid these issues is simply not practical at large scale and is expected to incur detrimental levels of stress to the pupae.

For these reasons, the irradiation at adult stage could be a more practical and reliable option for the bulk sterilization of mosquitoes. Most notably, water, and thus hypoxia would no longer be a variable factor. Perfectly synchronized larval rearing (to achieve pupation within a 24 h window) would also no longer be as critical (and limiting) issue, significantly easing the practicality and efficiency of the irradiation process. However, to irradiate adult mosquitoes in bulk, these require immobiliaztion by either chilling (Culbert et al., 2019; Zhang et al., 2020) or treatment with anesthetics, such as in nitrogen, carbon dioxide, argon, chloroform, desflurane, or other alternative chemicals.

To verify the notion that standardizing irradiation for adult mosquitoes is feasible, we investigated some factors that may affect dose-response in adult male mosquitoes in comparison to pupae. Previous reports by Helinski et al. (Helinski et al., 2006) and Du et al. (Du et al., 2019) have shown that in both *Anopheles arabiensis* and *Aedes albopictus* respectively, adults are slightly more radio sensitive than old pupae, although the difference was generally not statistically significant. As no recent reports cover the comparative radiosensitivity of adults and old pupae in *Ae. aegypti*, we first studied the dose response curves of both life stages in this species, and then assessed the effects of ambient temperature (chilling), and anoxia in *Ae. albopictus* adults.

2 MATERIALS AND METHODS

2.1 Mosquito Strains and Rearing

Standard laboratory reference strains of *Ae. aegypti* and *Ae. albopictus* (FAO/IAEA, 2017, 2020) were used for all experiments. The *Aedes* strains have been maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes" (FAO/IAEA, 2017).

2.2 Irradiation and Dosimetry

Radiation treatments were performed in a Gammacell 220 (Nordion Ltd., Kanata, Ontario, Canada), which had a dose-rate of 68 Gy/min during the temperature experiment (**Section 2.4**), and 65 Gy/min during the anoxia experiment (**Section 2.5**).

The dosimetry system used to verify the dose received by the samples was based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, United States) following the IAEA protocol (IAEA, 2004). Three films of either HD film (for doses >50 Gy) or MD film (for doses <50 Gy) were packed in small (2 × 2 cm) paper envelopes and placed directly above and below the mosquito samples. Films were read with an optical density reader after 24 h of development.

A diagnostic dose of 45 Gy was applied for most experiments, expecting to achieve around 95% sterility, to avoid 0 hatch results that cannot be usefully compared between treatments.

2.3 Assessing the Dose Response Curve for Pupal and Adult Stages of *Aedes aegypti*

Aedes aegypti were selected for this study as direct comparisons of pupal and adult radiosensitivity have not yet been reported in this species, contrary to *Ae. albopictus* (Du et al., 2019) and *An. arabiensis* (Helinski et al., 2006).

The doses for the dose-response curves for adult versus pupae of *Ae. aegypti* were selected according to the expected dose required to induce 50–100% sterility: 20, 55, 70, and 90 Gy.

Aedes aegypti eggs from one egg batch were collected and split in half to be hatched in two hatch events, 2 days apart (one for collecting adults, and one for collecting pupae for irradiation at the same time).

Adult males that emerged within an 8 h window were collected, batched in groups of 30, and kept in 15 × 15 × 15 cm Bugdorm® cages (MegaView Science Co. Ltd., Taichung 40762, Taiwan) until the following day when they were transferred to, and irradiated in small 2 cl plastic cups closed with a sponge. At the time of irradiation, the adults were 24–32 h old.

Pupae from the same cohort were collected in 4-h windows to ensure uniform pupal age of 40–44 h. We chose this age group as this represents the last hours before they begin to emerge into adults and are most radioresistant at this stage. The pupae were sexed based on pupal size dimorphism using a glass pupal sorter (Focks, 1980) and sex was verified under a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure virginity for later mating. Male pupae were counted into batches of 30 and were placed inside 2 cl plastic cups with excess water removed for irradiation.

Both the pupae and adults in each technical repetition were irradiated at the same time. Two biological repetitions and three technical repetitions were performed for all doses. Controls received the same handling but were not irradiated.

2.3.1 Assessment of Induced Sterility

Following irradiation, the male adults were placed in a 15 × 15 × 15 cm Bugdorm® cage, and pupae were placed in cups with water in separate cages for emergence. Thirty virgin females were added to each cage when the adults reached 2 days of age and were allowed to mate for 3 days before they were provided with 2 bloodmeals on consecutive days (days 6 & 7 post-emergence). Oviposition cups containing water and germination papers were added to each cage on day 8 for *en masse* egg collection (on days 9 & 10 post-emergence) following routine rearing protocols (FAO/IAEA, 2017). Egg papers were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. The total number of hatched and un-hatched eggs were counted using a stereomicroscope. Any non-hatched eggs were either opened with a dissection needle, or if many, were bleached to determine the fertility status (FAO/IAEA, 2019).

2.4 Effects of Chilling on Pupae and Adult Radiosensitivity, Flight Ability and Longevity in *Aedes albopictus* Irradiated as Adults

2.4.1 Dose-Response

As for the previous experiment, *Ae. albopictus* eggs from one egg batch were collected and split in half to be hatched in two hatch events, 2 days apart.

Adult males that emerged within an 8 h window were collected, batched in groups of 30, and were kept in 15 × 15 × 15 Bugdorm cages until the following day. The cages were then either kept at room (insectary) temperature (27° ± 2°C) ("rm temp") or were placed in a cold room for knock down at 5°C for 5 min, and then in a climatic chamber at 7°C ("chilled") for 1 h prior to the irradiation event. The treatments were thus either Control rm temp, or 45 Gy rm temp, or control chilled, or 45 Gy chilled. The adult males for the irradiation treatment were then transferred to 2 cl plastic cups closed with a sponge and were taken to the irradiator in Styrofoam boxes; the chilled males were kept in the cool box at 7°C until placed inside the GC220

FIGURE 1 | Irradiation set-up for adults irradiated in nitrogen or in air. One biological repetition with each three technical repetitions were irradiated simultaneously for each treatment, within a 5 mm thick PMMA container. The container was placed on a Styrofoam step so that all samples were in the middle of the irradiation chamber.

irradiator chamber and irradiated in same small 2 cl plastic cups closed with a sponge. At the time of irradiation, the adults were 2 days old.

Pupae from the same cohort were collected in an 8-hour window to ensure that all pupae were at least 36 h old during irradiation. The pupae were sexed based on pupal size dimorphism using a glass pupal sorter (Focks, 1980) and sex was verified under a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure virginity for later mating for both the adults and pupae treatment groups. Male pupae were counted into batches of 30 and were placed inside 2 cl plastic cups. The samples were subjected to the same treatments as described for adults above. The treatments were thus either Control rm temp, or 45 Gy rm temp, or control chilled, or 45 Gy chilled. Before irradiation, excess water was removed from the cups holding pupae. Two biological repetitions with each 3 technical repetitions were performed for each treatment.

Egg hatch rates were assessed as described in the above section "Assessing the dose response curve for pupal and adult stages following irradiation in a GC220/2.3.1 Assessment of induced sterility".

2.4.2 Longevity Under Mating Stress

Three of each treatments group and controls each, for both "adults" and "pupae" were kept in the cages post mating and oviposition to follow the longevity of the males. Dead males were counted and removed at least 4 times per week until all males were dead.

2.4.3 Flight Ability

One hundred male adults per treatment (rm temp or chill) and per repetition were collected from the same cohort and irradiated as described in the above section "dose response".

After irradiation, adults were allowed to recover for 2 days before they were taken to the flight test devices. Each batch of 100 males were placed inside the flight tubes for a duration of 2 h. Escaped and non- escaped adults were then counted as described by Culbert et al. (Culbert et al., 2018). Two repetitions with each 2 technical repetitions were completed.

2.5 Effects of Anoxia on Adult Dose-Response, Flight Ability and Longevity in *Aedes albopictus*

2.5.1 Dose-Response

Adult *Ae. albopictus* males that emerged within an 8 h window were collected, batched in groups of 20–30, and were kept in 15 × 15 × 15 cm Bugdorm cages until the following day. The batches of adult males for the "normoxic treatment" were then transferred to plastic "*Drosophila*" tubes (9 cm height × 2.7 cm diameter) closed with a sponge. The sponges were pushed down before irradiation so that the samples were in a similar position to the adults immobilized with nitrogen (**Figure 1**). Adult batches for the "anoxic treatment" were placed in gas tight glass head space vials (20 ml) with screw tops with PTFE/silicon septum (Merck KGaA, Darmstadt, Germany), additionally sealed with PTFE Thread Seal Tape (Sigma-Aldrich, United States). The oxygen was then replaced by nitrogen by adding nitrogen *via* a syringe needle (a second needle was inserted for outgoing gas), for 10 s, until all adult mosquitoes were immobile, and the 2 syringe needles were removed. Both anoxic and normoxic groups (3 technical repetitions each) were irradiated at 45Gy simultaneously (in alternating positions) in a 12 cm diameter PMMA container, in the GC220 irradiator (**Figure 1**). Three biological repetitions from different cohorts (with each 3 technical repetitions) were performed in total. At the time of irradiation, the adults were 2 days old.

Induced sterility was assessed as described in the above section "Assessing the dose response curve for pupal and adult stages following irradiation in a GC220/2.3.1 Assessment of induced sterility".

2.5.2 Flight Ability

One hundred male adults per treatment (normoxia (oxygen) or anoxia (nitrogen), irradiated at 45 Gy, and non-irradiated controls) and per repetition were collected from the same cohort and irradiated as described in the above section "dose-response".

Flight tests were performed as described in the above section "**Section 2.4** and **Section 2.4.3**". Two repetitions with each 3 technical repetitions were completed.

2.5.3 Longevity Under Mating Stress

All treatments groups and controls were kept in the cages post mating and oviposition to follow the longevity of the males. Dead males were counted and removed at least 4 times per week until all males were dead. Three repetitions were done for all treatment groups and controls for both treatment groups irradiated as pupae and as adults.

2.5.4 Longevity Following High Doses- Males Only

As little difference was seen following the previous longevity experiments, and mating stress is known to decrease survival in males, an additional experiment was added to assess the effects of anoxia on sterile male longevity, without the added factor of mating stress. For this, additional batches of 30 males were irradiated in either normoxia (oxygen) or anoxia (nitrogen) as described in the previous section. All males were over-dosed at 90 Gy (beyond the fully sterilizing dose) in the GC220 as described above. The males were then returned to 15 × 15 × 15 cm Bugdorm cages and dead males were counted and removed at least 4 times per week until all males were dead.

2.6 Statistical Analysis

All statistical analyses were performed in R (version 4.1.0) using RStudio (RStudio, Inc. Boston, MA, United States, 2016). Generalized Linear Mixed Models (GLMM, lme4 package) were used with the appropriate distribution family.

To analyze the dose response curve of pupae versus adults for *Ae. aegypti*, a binomial GLMM fit by maximum likelihood (Laplace Approximation) was used for egg hatch rates considered as response variable, life stage (2 levels: pupae and adults), irradiation log (dose) (4 levels: 20, 55, 70 and 90 Gy) and their interaction were considered as fixed effects and the repetition as a random effect.

For the effects of chilling on pupae and adult radio-sensitivity in *Ae. albopictus*, a binomial GLMM was also used with egg hatch rates as response variable, treatment (2 levels: room, chilling), life stage (2 levels: pupae and adults), irradiation dose (2 levels: 0 and 45 Gy) and their interaction considered as fixed effects and repetition nested with technical repetition as a random effect.

Similarly, male flight ability data was analyzed as response variable, treatment (4 levels: Chilled/room temperature, irradiated/non-irradiated; or anoxia/normoxia and irradiated/non-irradiated) as fixed effect and the repetition nested with technical repetition as a random effect considering each specific experiment.

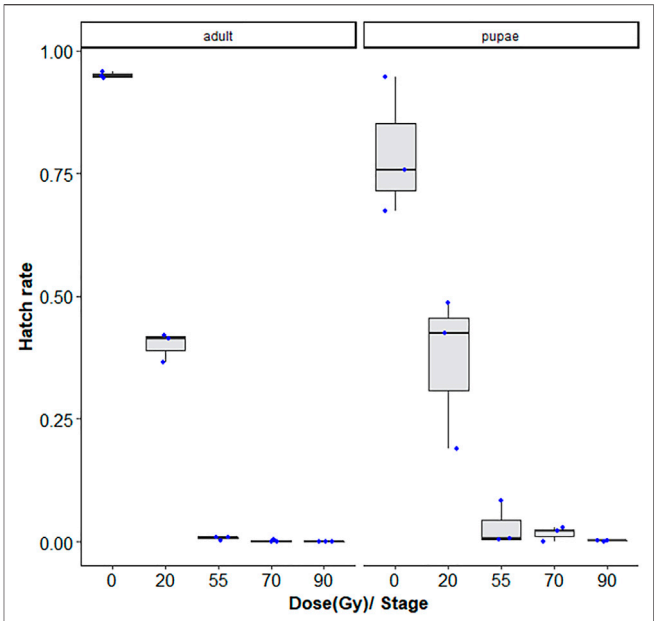

FIGURE 2 | Dose-response shown as egg hatch rate of *Aedes aegypti* pupae vs. adults. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

Mixed Effects Cox Models ("coxme" function in 'survival' package) fit by maximum likelihood with mosquito time to death as response variable, treatment (4 levels: chilled, room temperature, irradiated, non-irradiated; or 3 levels: anoxia, normoxia, non-irradiated control) and their interaction as fixed effects and repetition as random effect, were used to analyze the survival of mosquitoes following the treatment in each specific experiment. Survival graphs were built using the packages "survival," "ggplot2," and "ggpubr".

The full models were checked for overdispersion using Bolker's function (Bolker and R Development Core Team, 2020) (in package bblme). The best model was chosen based on the lowest AICc s and models were simplified using the stepwise removal of terms, followed by likelihood ratio tests (LRTs) when appropriate. Multiple comparisons using the "emmeans" function (in package "emmeans") (https://github.com/rvlenth/emmeans) were performed between the levels where significant differences were found. A p-value of less than 0.05 was used to indicate statistical significance in all cases.

3. RESULTS

3.1 Dosimetry

The dosimetry confirmed that all doses received laid within a 5% error range.

TABLE 1 | Fixed effects of chilling on pupae and adult radiosensitivity.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	2.0095	0.1826	11.005	<2e-16 ***
Room temperature	–0.3408	0.1342	–2.539	0.0111 *
Pupae stage	0.2332	0.1315	1.773	0.0762
Dose45Gy	–4.6976	0.1651	–28.446	<2e-16 ***

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

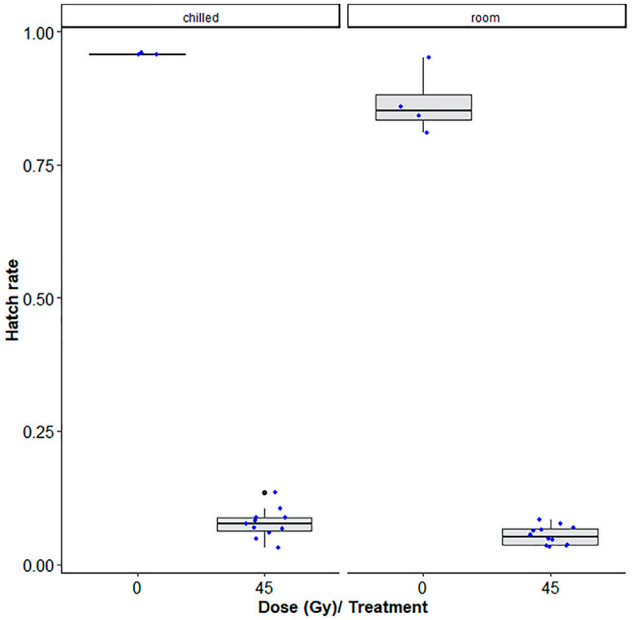

FIGURE 3 | Dose-response shown as egg hatch rate of *Aedes aegypti* pupae vs. adults. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

3.2 Assessing the Dose Response Curve for Pupal and Adult Stages of *Aedes aegypti*

As expected, the hatch rate reduced signficantly with the dose (GLMM: χ^2 = 2,589, df = 2, $p < 0.001$). For 20, 55, 70, and 90 Gy doses tested, adults were more radiosensitive than the late stage pupae, with <1–~3% lower fertiliy levels following radiation exposure (GLMM: χ^2 = 52.685, df = 2, $p < 0.001$, **Supplementary Table S1**). There was also a higher degree of variation observed between repetitions for the pupae samples (**Figure 2**).

3.3 Effects of Chilling on Pupae and Adult Radiosensitivity, Flight Ability and Longevity in *Aedes albopictus* Irradiated as Adults

3.3.1 Dose-Response

Table 1 shows that room temperature led to lower hatch rates as compared to chilling, i.e., chilling led to decreased induced sterility (GLMM: χ^2 = 6.4454, df = 1, p = 0.0111, **Figure 3**) while no difference was observed between pupae and adult *Ae. albopictus* mosquito stages (χ^2 = 3.1432, df = 1, p = 0.07625). Additionally, irradiation dose of 45Gy significantly reduced the egg hatch rates (χ^2 = 809.1845, df = 1, $p < 0.001$, **Figure 3**).

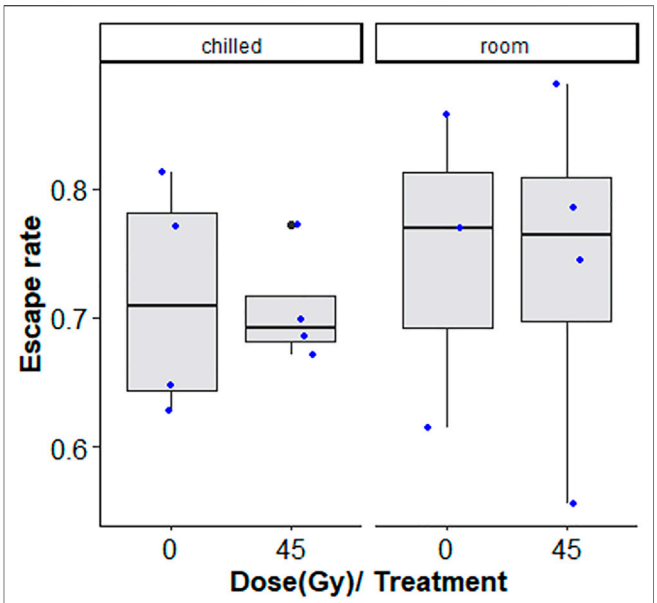

FIGURE 4 | Flight ability of chilled versus non-chilled, sterile *Aedes albopictus* male adults irradiated at 45 Gy or not irradiated The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

TABLE 2 | Fixed effects of chilling and irradiation on flight ability in *Aedes albopictus males*.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	0.85361	0.09806	8.705	<2e-16 ***
Room temperature	0.15859	0.10535	1.505	0.132
Dose45Gy	0.04216	0.09957	0.423	0.672

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

TABLE 3 | Fixed coefficients of the effects of pupae chilling on male *Ae. albopictus* longevity.

	Coef	exp (coef)	se (coef)	z	*p*
Dose	0.001195	1.001196	0.004363	0.27	0.78
Doom temperature	0.05391	1.055389	0.24482	0.22	0.83
Dose:room temperature	–0.003	0.997009	0.006248	–0.48	0.63

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1*

3.3.2 Effects of Chilling on Flight Ability

Chilling for 1 h at 7°C, with or without irradiation at 45 Gy followed by 2-day-recovery had no negative effects on flight ability (**Figure 4**; **Table 2**).

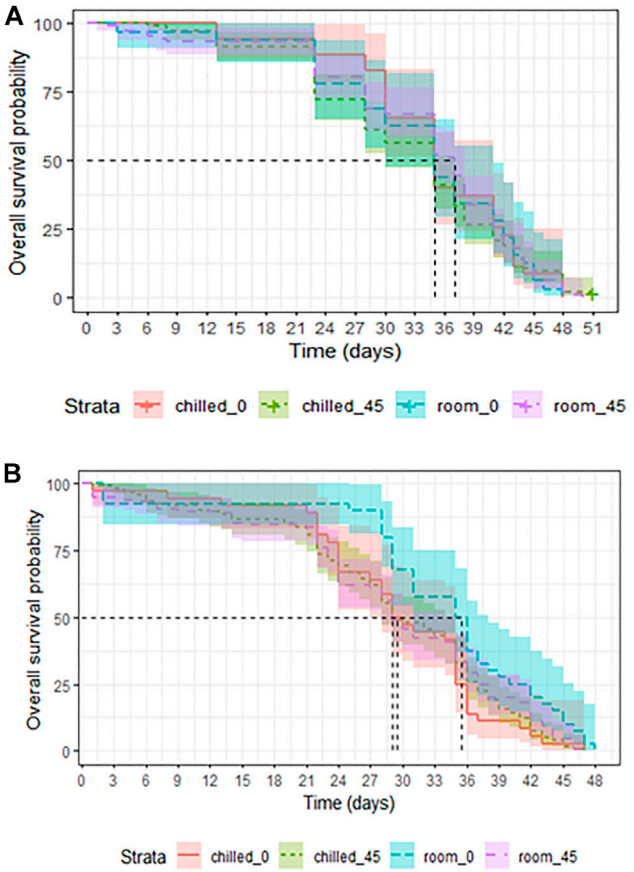

FIGURE 5 | Longevity of chilled versus non-chilled *Aedes albopictus* males irradiated at 45 Gy as either pupae **(A)** or as adults **(B)**, compared to non-irradiated controls.

TABLE 4 | Fixed coefficients of the effects of adult chilling on male *Ae. albopictus* longevity.

	coef	exp (coef)	se (coef)	z	p
Dose45Gy	0.210645	1.234474	0.138563	1.52	0.13
Room temperature	−0.19772	0.820597	0.11508	−1.72	0.086

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

3.3.3 Effects of Chilling on Male Longevity

In adults that were chilled and/or irradiated as late pupae, survival was not affected by chilling (LRT: $\chi^2 = 0.1582$, df = 1, $p = 0.6908$, **Table 3**), nor by irradiation with 45Gy (LRT: $\chi^2 = 0.0006$, df = 1, $p = 0.9383$, **Table 3**). Adults that were chilled and irradiated as pupae also lived as long as untreated controls (LRT: $\chi^2 = 0.224$, df = 1, $p = 0.6317$, **Table 3** and **Figure 5A**).

In mosquitoes that were chilled and/or irradiated at the adult stage, chilling had no negative effect on the overall longevity of adults (LRT: $\chi^2 = 2.947$, df = 1, $p = 0.08603$, **Table 4**). Irradiation at 45 Gy also did not affect overall longevity as compared to non-irradiated adults (LRT: $\chi^2 = 2.2893$, df = 1, $p = 0.1303$, **Table 4**). Adults that underwent chilling plus irradiation at 45 Gy were marginally negatively impacted as compared to untreated controls (LRT: $\chi^2 = 3.39$, df = 1, $p = 0.06559$, **Figure 5B**). Chilling, and combined chilling and irradiation treatments reduced the median survival from the 35.5 (31-38, 95%CI) days in the control group to 29.0–29.5 (28-35, 95%CI) days (**Supplementary Table S2**), however, the difference was not significant.

TABLE 5 | Fixed effect of anoxia on adult dose-response.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	4.3298	0.1959	22.105	<2e-16 ***
Dose45Gy	−5.2631	0.1317	−39.954	<2e-16 ***
Normoxia	−1.0435	0.1425	−7.323	2.43e-13 ***
Dose45Gy:rormoxia	−0.8462	0.1546	−5.474	4.39e-08 ***

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

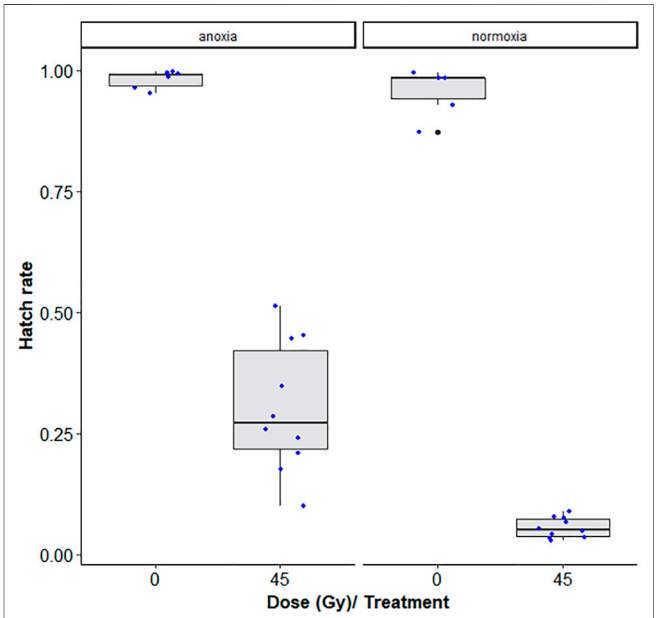

FIGURE 6 | Egg hatch rates of *Aedes albopictus* males irradiated at 45Gy in normoxia (O_2) vs. anoxia (N_2). The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

3.4 Effects of Anoxia on Adult Dose-Response, Flight Ability and Longevity in *Aedes albopictus*

3.4.1 Dose-Response

There was a significant interaction between anoxia and irradiation dose effects ($\chi^2 = 29.968$, df = 1, $p < 0.001$, **Table 5**). Adult males irradiated at 45 Gy in anoxia were on average 5.7 times more fertile than those irradiated in normoxia ($p < 0.001$, **Supplementary Table S3**; **Figure 6**). The highest observed difference in fertility was a 14-fold difference between samples irradiated in anoxia versus normoxia. More variability

TABLE 6 | Fixed effects of irradiation in anoxia on flight ability.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	1.922925	0.151896	12.66	<2e-16 ***
Atmnormoxia	0.605234	0.178105	3.398	0.000678 ***
Dose45Gy	−0.00993	0.177462	−0.056	0.955386

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

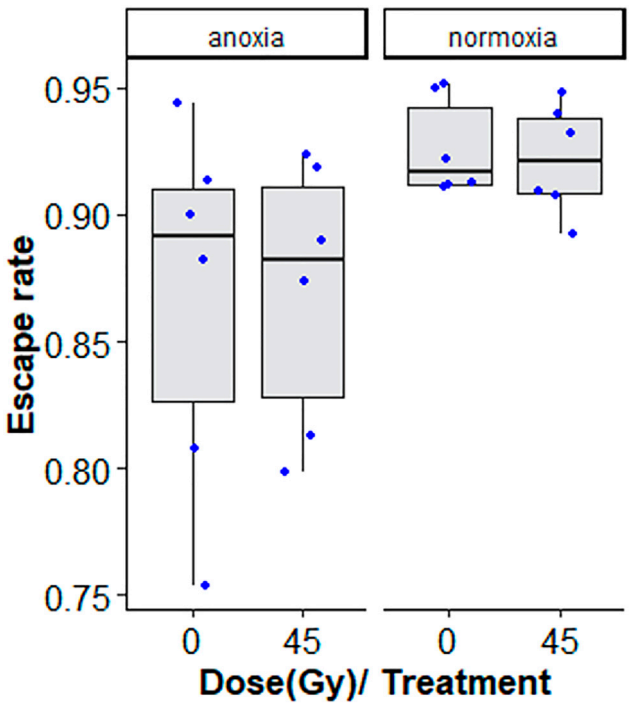

FIGURE 7 | Flight ability of adults treated in nitrogen [non-irradiated controls (N_2C) and irradiated (N_{2i})] compared to adults in air [non-irradiated controls (O_2C) and irradiated in air (O_{2i})]. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

within samples and between technical repetitions were also observed in the anoxia treated groups as compared to the normoxic groups.

3.4.2 Effect of Irradiation in Anoxia on Flight Ability

Treatment with nitrogen (anoxia) negatively affected flight ability, regardless of whether irradiated or non-irradiated (GLMM: χ^2 = 29.642, df = 1, p < 0.001, **Table 6** and **Supplementary Table S4**). Irradiation at 45 Gy did not reduce flight ability, neither in the anoxia treatment groups), nor in the normoxic (oxygen) groups (GLMM: χ^2 = 0.0829, df = 1, p = 0.7734, **Figure 7**; **Supplementary Table S4**). Again, results were much more variable in groups subjected to anoxia.

3.4.3 Longevity Under Mating Stress

A significant interaction between dose and atmosphere was observed (LRT: χ^2 = 19.427, df = 1, p < 0.001). However, when

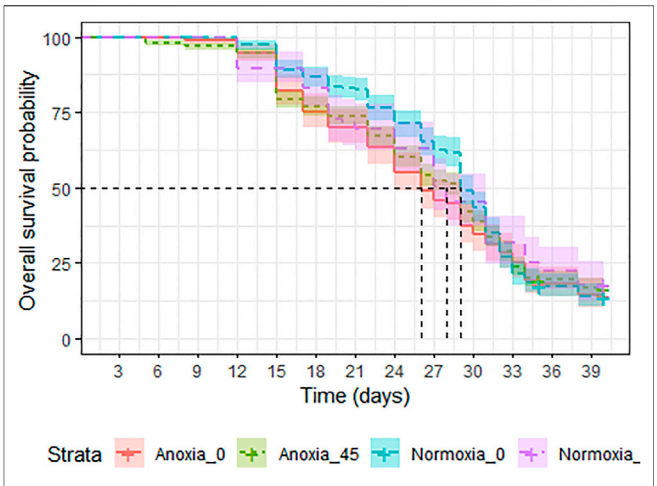

FIGURE 8 | Longevity under mating stress of adult *Aedes albopictus* males irradiated at 45 Gy in either anoxia (N_2) or normoxia (O_2) compared to untreated controls.

comparing each treatment group, anoxia had a negative effect on survival in the non-irradiated groups (Odd ratio = 1.269, z. ratio = 2.652, p = 0.04, **Supplementary Table S5**), but decreased the risk of mortality in the irradiated groups (Odd ratio = 0.631, z. ratio = −3.793, p = 0.0009, **Supplementary Table S5**), when males were caged with females at a 1:1 ratio and were assessed under mating stress (**Figure 8**). Between the normoxic groups, irradiation with 45 Gy reduced the longevity slightly (Odd ratio = 0.593, z. ratio = −4.146, p = 0.0002, **Supplementary Table S5**). Median survival was 29 (95%CI 29–30) days for untreated controls, and for the goups irradiated in anoxia (95%CI 27–29). Groups irradiated in normoxia and groups treated with only anoxia showed a slightly reduced median survival time of 28 (95%CI 27–31) and 26 (95%CI 27–29) days, respectively (**Supplementary Table S6**).

3.4.4 Longevity Following High Doses- Males Only

Treatment had a significant effect on adult survival (LRT: χ^2 = 164.9, df = 2, p < 0.001). Longevity of males irradiated in anoxia was not affected compared to untreated controls (p = 0.054, **Table 7**; **Figure 9**) even when the dose was doubled to 90 Gy, wheras males irradiated in normoxia at the same dose were highly negatively impacted (p < 0.0001, **Table 7**; **Figure 9**). The median survival of the untreated controls and the adults irradiated with 90 Gy in anoxia was 43 (95% CI 40–43) and 41 (95%CI 41-41) days respectively, wheras adults irradiated with 90 Gy in normoxia had a median survival of 28 (95%CI 28-28) days (**Supplementary Table S7**).

4 DISCUSSION

The series of experiments reported here have shown that there are various factors that affect dose response in mosquito adults, which need to be taken into consideration when developing

TABLE 7 | Fixed coefficients of the effects of anoxia with high doses on longevity for males only.

	coef	exp (coef)	se (coef)	z	p
Anoxia_90Gy	−0.19949	0.819147	0.103508	−1.93	0.054
Normoxia_90Gy	1.034523	2.813763	0.102299	10.11	0

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

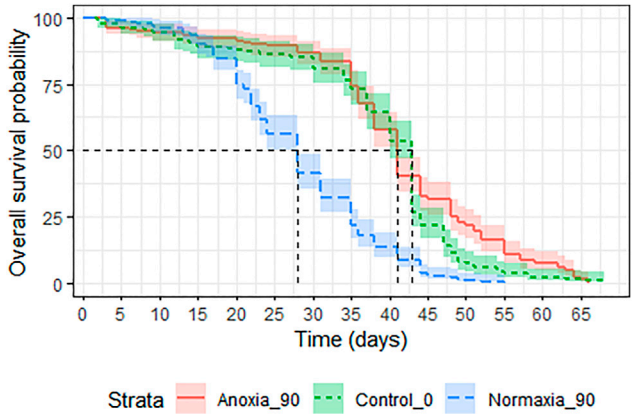

FIGURE 9 | Longevity of adult male *Aedes albopictus* irradiated at high dose (90 Gy) in normoxia (O_2) or anoxia (N_2) compared to untreated controls.

irradiation protocols for adults in the frame of the SIT. These factors not only affect irradiation outcome in terms of sterility levels achieved in the males, but also downstream quality parameters important for male performance once released.

Adult *Ae. aegypti* mosquitoes were slightly more radiosensitive than late-stage pupae. However, one must consider that the younger the pupae, the more sensitive and the more prone to somatic damage (Yamada et al., 2019). In general, *Aedes* pupae, just before emergence seem to be at their most resistant phase to various treatments, such as chilling, desiccation, hypoxic environments and irradiation contrarily to (some) fruit flies, where the pupae are most sensitive on the day before emergence when they are undergoing extensive mitotic divisions for the buildup of the adult organism (Economopoulos, 1977). The dose-response curves for pupae versus adult *Ae. aegypti* corroborate those reported for *An. arabiensis* (Helinski et al., 2006) and *Ae. albopictus* (Du et al., 2019), where adults were slightly more sensitive, but for the most part, the difference was not significant, as was seen in the comparison of late-stage pupae and adult *Ae. albopictus* in the second experiment. The homogeneity of sterility levels within and between adult samples was better than in pupae samples, although pupal age was carefully controlled. Considering that adults do not require higher doses, and the high levels of consistency seen within and between irradiated batches are advantages of irradiating at this stage, in addition to facilitated timing and ease of handling for irradiation exposures.

The next factor that requires scrutiny is the immobilization of adults during bulk irradiation needed for operational programmes. Therefore, assessing the effects of chilling on dose-response in terms of sterility and downstream male performance was essential. Many studies are available in which cold temperatures reduced insect flight ability, or mating competitiveness (Mutika et al., 2002; Shelly et al., 2010; Reynolds and Orchard, 2011; Andress et al., 2014; Diallo et al., 2019), or where cold treatment was used to enhance phytosanitary practices (Gould and Sharp, 1990; von Windeguth and Gould, 1990; Burikam et al., 1992; Follett and Snook, 2013; de Kock and Holz, 2017). However, in these reports, cold treatments were applied separately from the irradiation step, and the direct effect of chilling on dose response was not investigated.

Andress et al., (Andress et al., 2012) found that chilling (3–8°C for 2–6 h) decreased flight ability dramatically in the Mediterraenan fruitfly (*Ceratitis capitata*), whereas Tanahara and Kirihara (Tanahara and Kirihara, 1989), and Reynold and Orchard (Reynolds and Orchard, 2011) found no detrimental effects of chilling on this parameter in the melon fly (*Bactrocera curcurbitae*) and Qeensland fruitfly (*B. tryoni*) respectively. Shelly et al. (Shelly et al., 2010) observed negative effects of chilling on the flight capacity and mating performance of *C. capitata* held at high densities, however, the mating performance was restored after 3 days of recovery. A recovery of host-searching abilities of the parasitoid *Diachasmimorpha longicaudata* following damages from chilling for packaging was also observed after 1–2 days (Cancino et al., 2020). In tsetse flies, Diallo et al. (2019) reported that chilling was one of the main factors negatively affecting the quality of the sterile flies, in terms of emergence rates and flight ability. This corroborated findings of Mutika et al. (2002), who reported a decrease in insemination rates and a dramatic increase in mortality of adult *Glossina pallipides* following low temperature (7 and 4°C)treatment of pupae.

There are numerous other studies that evaluate the effects of temperature on insect quality, however few exist that assessed cold temperature effects during radiation exposures on the irradiation outcome itself. Most studies investigated its direct effects on fertility, or the downstream effects of the combined irradiation and chilling on sterile male quality. For instance, a decrease in survival of adult *G. morsitans* was reported as irradiation dose was increased, and this decline was more pronounced in cohorts irradiated at low temperatures (2°C) (Curtis and Langley, 1972). Langley and Maly (1971) also found chilling to have deleterious effects on adult emergence rates and adult male survival in the fruitfly *C. capitata*, and proposed oxygen-dependent effects of irradiation to be the cause, due to higher oxygen saturation levels at lower temperatures.

Chilling *Ae. albopictus* at 7°C before and during irradiation had no negative impact on longevity when treated as pupae, and only resulted in a marginal reduction in longevity when treated as adults (though not statistically significant). However, the chilling conditions of the present study did slightly reduce the sterility levels achieved as compared to males that did not undergo chilling treatments, but only by a few percent (~3%). This implies that the cold temperature had some degree of radioprotective effect during the exposure. Here, it was again observed that the variation in sterility levels was higher in and

between pupae samples and adult samples were more homogeneous, as was seen with *Ae. aegypti* in the first experiment in this report.

Culbert et al. (2019) studied the effects of chilling in mosquito adults on quality control (QC) parameters, and found that chilling had negative effects on the survival in *Ae. aegypti* and *Ae. albopictus*, where the latter was more sensitive to the cold treatment at all tested temperatures. Contrarily, chilling (at 2,4,6 and 10°C) for up to 8 h had no effect on the survival of *An. arabiensis* for 14 days. Only chilling at 2°C for 24 h resulted in a decrease in longevity in this species. Zhang et al. (2020) found the optimum chilling temperature and duration for *Ae. albopictus* to be 5–10°C for 3 h, resulting in no adverse effects on longevity and mating competitiveness.

Following a similar trend, it was found that chilling did not significantly reduce flight ability, although chilled irradiated groups showed marginally reduced escape rates (though not significant), compared to the unirradiated controls. There was no significant difference between chilled and non-chilled, unirradiated controls, due to a high variation in escape rates in both groups. It is possible that sufficient recovery of chilled adults occurs within the 2 days before the flight test. Significant decrease in flight ability was seen directly after chilling, but near full recovery was observed after 1–2 days in *Ae. aegypti* (Maiga, unpublished results).

The slight reduction in the sterility following irradiation in cold temperatures may be the consequence of the reduced metabolic rates in the mosquitoes, whereas the slight increase somatic damage leading to marginally reduced adult quality in the parameters assessed, and chilling induced damages as reported in other studies (referenced above) may be explained by higher oxygen saturation in the low temperature, leading to an increase in oxygen-dependent effects of irradiation, as proposed by Langely and Maly (Langley and Maly, 1971). In any case, it is known that both radiation damage as well as recovery are temperature-dependent and are both slow in cold temperatures (Sazykina and Kryshev, 2011).

There is a threshold for different insect species at which cold temperatures start to cause negative effects on the organism. For this reason, available studies present either negative or no effects, and seldom positive effects regarding sterile insect quality in the frame of the SIT. The slight radioprotective effects of irradiation in cold temperatures as seen here does not present added value in terms of improving mosquito quality or the SIT, other than its practicality of immobilizing and handling the adult mosquitoes. On the contrary, the degree of chilling and duration is important as it can induce negative effects if not controlled carefully. Therefore, it is worth investigating other methods for immobilization that may improve sterile male quality and irradiation procedures.

Nitrogen can also be used to immobilize mosquitoes for handling and irradiation processes, and its protective effects in insect irradiation have been known since 1947 (Thoday and Read, 1947) and has been widely reported for a variety of insect species.

Hypoxic conditions during insect irradiation have also been shown to often improve insect biological quality, even though higher doses are then needed to reach the desired sterility levels (Economopoulos, 1977; Ohinata et al., 1977; Rananavare et al., 1991). This is because the magnitude of the protective effects seems to be greater for somatic damage than for the induction of sterility (Bakri et al., 2021). For this reason, hypoxic conditions are often used to improve sterile insect quality without reducing their sterility levels (Lance and McInnis, 2005). However, some agents used to create hypoxic environments, such as CO_2 and N_2 are reported to have their own negative side effects, some of which disappear again after allowing a period of recovery (Birkenmeyer and Dame, 1970), and some with lasting effects. Other studies on irradiation in anoxia report great improvements on several quality parameters such as longevity, developmental parameters and mating performance (Baldwin and Salthouse, 1959; Baldwin and Chance, 1970; Langley and Maly, 1971; Curtis and Langley, 1972; LaChance and Richard, 1974; Fisher, 1997).

Only few reports exist where irradiation of mosquitoes in nitrogen is described, and effects on quality parameters are assessed. El-Gazzar (1983) exposed *Culex quinquefasciatus* to radiation in a nitrogen atmosphere and showed the reduced effects on sterility induction but found little to no improvement on mating performance. Hallinan and Rai (Hallinan and Rai, 1973) reported that for low doses, nitrogen improved mating competitiveness in *Ae. aegypti*, compared to males irradiated in air, similar to what Terwedow and Asman (Terwedow and Asman, 1977) reported for *Ae. sierrensis*, but none of the publications describe any improvement in other male quality parameters.

Our study also showed that hypoxia protects from O_2 effects during adult irradiation as was seen in mosquito pupae (Yamada et al., 2019, 2020), but may come with its own negative effects. Anoxia had high radioprotective effects, with up to a 14-fold increase in residual fertility compared to males irradiated with the same dose in normoxia. Anoxia did not have an effect on fertility in unirradiated controls. However, the treatment with N_2 itself had a negative impact on flight ability. The 2-day recovery time allowed males that were irradiated only, to fully recover flight ability, whereas those treated in N_2 were unable to recover within this time frame, whether irradiated or not. This implies that the treatment with N_2 was more important for the reduced flight capacity than the irradiation treatment with 45 Gy. It is possible that a longer recovery time could restore flight ability but storing sterile males for much longer than 2–3 days post irradiation may decrease efficiency in the SIT programmes, where space and extra days of handling are costly.

There was a slight reduction in longevity when adult males were irradiated with 45 Gy in normoxia or anoxia, when they were caged with females at a 1:1 ratio. However, when males were caged alone, and were not subjected to mating stress, those males overdosed with 90 Gy in anoxia survived significantly better than the males irradiated with the same dose in normoxic conditions. We suggest that either 45 Gy is a low enough dose in this species to not see a large effect on longevity [as seen in other reports (Balestrino et al., 2010)], or males irradiated in anoxia are not only more fertile, but also more virile, mating more, and thus slightly reducing longevity in the mixed sex cages, contrary to the results seen at the higher dose, but where females were absent. Males overdosed with 90 Gy in nitrogen lived significantly longer

that males irradiated in oxygen and the protective effects of anoxia were clearly observed. To better understand the meaningfulness of longevity studies, it would be important to further examine the effects of the various study designs and variables, such as cage size and adult density, and the inclusion of females (at various ratios) to observe the magnitude of mating induced stress and its effects on male survival. In this study, the males caged alone generally survived more than 2 weeks longer than the males caged with females, suggesting that mating stress has considerable effects on survival and can mask effects of other treatments that are actually the focus of the study.

Although nitrogen had radio protective effects which may preserve fertility and longevity, it seems that treatment with nitrogen in general (with or without the additional radiation exposure) had negative effects on male flight ability, and potentially other parameters which may be the more important factors for mating success in the wild. The full effects of anoxic treatments need to be carefully assessed in field cage mating studies.

There is a need for anesthetics for insect immobilization for facilitating handling. However, many reports have shown that immobilizing agents induce negative side effects (Crystal, 1967; Birkenmeyer and Dame, 1970), the extent of which depends on the sex and age of the insect, as well as the duration and frequency of exposures to the various gases, similar to treatments in cold temperatures. It is therefore necessary to carefully assess these factors and all available options before formulating protocols for mosquito immobilization and handling.

5 CONCLUSION

These experiments gave an initial indication of factors that affect dose-response in mosquito adults, especially in terms of sterility achieved, and downstream effects of chilling and anoxia on selected male quality parameters. Both methods present advantages and disadvantages and affect some quality parameters positively and others negatively. It is important to note that the irradiation dose needs to be adjusted to achieve the desired level of sterility, before considering treatment protocols that could improve sterile male quality for SIT programmes. Additionally, available immobilizing techniques for improved handling need careful evaluation and balance between practicality and potential costs to insect quality to ensure there is a clear benefit before their application in the field.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HY conceptualized the experimental designs for the experiments, caried out the experiments and drafted the original manuscript. HM carried out the flight tests and contributed significantly to the written content and later versions of the manuscript. AA contributed to the design of the anoxia setup and experiment and provided equipment and materials. CK, WM, NB, and TW provided all live material following standardized rearing procedures and assisted in the experiment and data collection. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.856780/full#supplementary-material

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Intronic gRNAs for the Construction of Minimal Gene Drive Systems

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Gene drives are promising tools for the genetic control of insect vector or pest populations. CRISPR-based gene drives are generally highly complex synthetic constructs consisting of multiple transgenes and their respective regulatory elements. This complicates the generation of new gene drives and the testing of the behavior of their constituent functional modules. Here, we explored the minimal genetic components needed to constitute autonomous gene drives in *Drosophila melanogaster*. We first designed intronic gRNAs that can be located directly within coding transgene sequences and tested their functions in cell lines. We then integrated a Cas9 open reading frame hosting such an intronic gRNA within the *Drosophila rcd-1r* locus that drives the expression in the male and female germlines. We showed that upon removal of the fluorescent transformation marker, the *rcd-1r*^{d} allele supports efficient gene drive. We assessed the propensity of this driver, designed to be neutral with regards to fitness and host gene function, to propagate in caged fly populations. Because of their simplicity, such integral gene drives could enable the modularization of drive and effector functions. We also discussed the possible biosafety implications of minimal and possibly recoded gene drives.

Keywords: gene drives, genetic control, *Drosophila*, synthetic biology, genetics

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INTRODUCTION

Synthetic gene drives are designed to be transmitted to the progeny at super-Mendelian (>50%) frequencies. CRISPR–Cas9-based gene-drive systems have recently been shown to propagate efficiently in laboratory populations of several insects (Raban et al., 2020; Bier, 2022). They are thus seen as novel tools to modify wild populations of organisms, offering new strategies to reduce the impact of vector-borne diseases and eliminate populations of agricultural pests or target invasive species. Arthropod-borne diseases remain at the forefront of gene drive research. They are endemic in more than 100 countries and affect approximately half of the world's population (Hill et al., 2005). Mosquitoes are the vectors of several diseases of major global public health importance, including malaria and dengue fever. Although the use of currently available malaria control tools over the past 2 decades has greatly reduced malaria cases and deaths, progress has decreased in recent years (WHO, 2021), and eliminating malaria will likely require new development technologies and tools. The dengue virus is a potential threat to an estimated 2.5 billion people, and the last half-century has witnessed a 30-fold increase in the global incidence of dengue (Harapan et al., 2020). Gene drive technology has reached a stage where constructs are capable of reliably eliminating caged mosquito populations (Hammond et al., 2016; Kyrou et al., 2018; Simoni et al., 2020; Hammond et al., 2021a; Hammond et al., 2021b). Gene drives for population replacement have also advanced, and proof of

principle has been achieved in mosquito vector species (Gantz et al., 2015; Pham et al., 2019; Adolfi et al., 2020; Carballar-Lejarazu et al., 2020). In addition to these advances, several innovative gene drive designs have been explored in model organisms focused on reducing the rise of resistance or to curtail gene drive spread (Champer et al., 2018; Oberhofer et al., 2019; Champer et al., 2020a; Champer et al., 2020b; Oberhofer et al., 2020; Price et al., 2020).

One approach of interest is the modularization of gene drive functions into a split drive or autonomous/non-autonomous gene drive components which can have advantages and which does not, in itself, reduce the level of the drive (Champer et al., 2019; Nash et al., 2019; Edgington et al., 2020; Kandul et al., 2020; Terradas et al., 2021). It allows for testing individual components of a gene drive strategy, for example, antimalarial effector molecules that require evaluation at a scale large enough so that the detection of expected or unexpected entomological and epidemiological effects is possible before they are widely applied (James et al., 2018). Modularization could allow to safely test such molecules at an ever larger scale moving safely and in defined steps from the laboratory to the field without a strict requirement for geographical isolation. For example, inundative releases of a non-autonomous effector-carrying strain would allow the assay of mosquito fitness and performance under field conditions and detection of any unintended effects prior to deployment. When tested in the absence of an active gene drive element, a non-autonomous effector will not convert the field population, permitting its safe testing (Nash et al., 2019). The release of the same non-autonomous effector strain in combination with a non-driving source of Cas9 could trigger a limited and localized spread of the effector trait and allow the evaluation of its drive performance and perhaps its epidemiological effect. At a later stage, full gene drive of the same effector traits could be enabled by introducing autonomous Cas9 drive elements. Modular gene drives could also be simpler, and each component is designed to feature only the minimal set of genetic modifications needed to be introduced into individual genomes to only those components necessary for global health, agriculture, or ecology. Along these lines, we have been developing a gene drive approach termed as the integral gene drive (IGD), which involves minimal genetic modifications of host genes and a separation of transmission blocking and gene drive functions into separate loci and strains (Nash et al., 2019). We have already shown that effector molecules can be expressed within endogenous mosquito loci and that those can be mobilized into non-autonomous gene drives when a source of Cas9 is provided in trans (Hoermann et al., 2021).

In the current study, we sought to design and test the other necessary component of such a system, i.e., an autonomous integral gene drive capable of expressing Cas9 and a gRNA using the *Drosophila* model. Different design criteria apply to this strategy which requires the modification of germline loci and the hosting of the substantial Cas9 coding sequence within such a locus. The sole purpose of such an autonomous drive construct is to propagate and seed Cas9 within a population while ideally having a minimal impact on the fitness or fertility of the target organism. The latter requires that the gRNA be expressed efficiently from within the same locus and does not interfere with either Cas9 or the host gene function. To achieve this, we designed intronic gRNA modules that could be located within coding sequences of other genes without interfering in their functioning and tested them in *Drosophila* S2 cells. One successful design was then further evaluated in transgenic flies and on the population level.

RESULTS

Establishing a Testing Platform for Intron-Encoded gRNAs

We first designed a synthetic gene circuit where the successful GFP expression was coupled to the activity of an intron-encoded gRNA (**Figure 1A**). Specifically, this circuit was designed so that both the splicing of the gRNA-containing intron (located within the reporter gene) and the efficient expression of the gRNA cassette itself were necessary to achieve high levels of eGFP expressions. For this purpose, we used a set of three gRNAs that had previously been described to be capable of recruiting a dCas9 transcriptional activator to the ×5 QUAS sequence (Lin et al., 2015). In this plasmid-based system, we provided the dCas9-VPR-T2A-mCherry activator together with a reporter plasmid containing the 5xQUAS motif upstream of a minimal Hsp70 promoter and the eGFP cassette which in turn hosted the intron constructs to be tested. We assessed two intron variants based on either the *Drosophila melanogaster ftz* intron or a synthetic intron previously characterized in a mammalian cell culture (Kiani et al., 2014). The QUAS-targeting gRNAs were incorporated into the introns upstream of the branch point, as summarized in **Figure 1B**. We either included the full U6:3 promoter and a T6 terminator or simply the gRNA sequence itself without any additional regulatory sequences. In the former case, we expected that the gRNA expression would be driven independently from the expression of the GFP gene whereas in the latter case, the spliced intronic RNA would itself act as a gRNA, as has been shown before (**Figure 1B**). A third variant was also generated where the gRNA was located directly on the 5′ strand of the poly-pyrimidine stretch where the leading T nucleotides were meant to act to terminate transcription.

Testing the Performance of Intron-Encoded gRNAs in *Drosophila* S2 Cells

The reporter and activation constructs were co-transfected into *Drosophila melanogaster* S2 cells to evaluate the performance of gRNA constructs and to simultaneously enable splicing and the transactivation of the eGFP expression. GFP fluorescence was quantified to determine the relative efficacy of each intron-encoded gRNA variant. As a negative control, we employed the *ftz* and synthetic intron base constructs prior to the insertion of any gRNA cassette. As a positive/activation control, we used these base constructs together with the dCas9-VPR activator into which we cloned a cassette co-expressing transactivating gRNA1. As expected, we detected, for both intron variants, low levels of the eGFP expression in

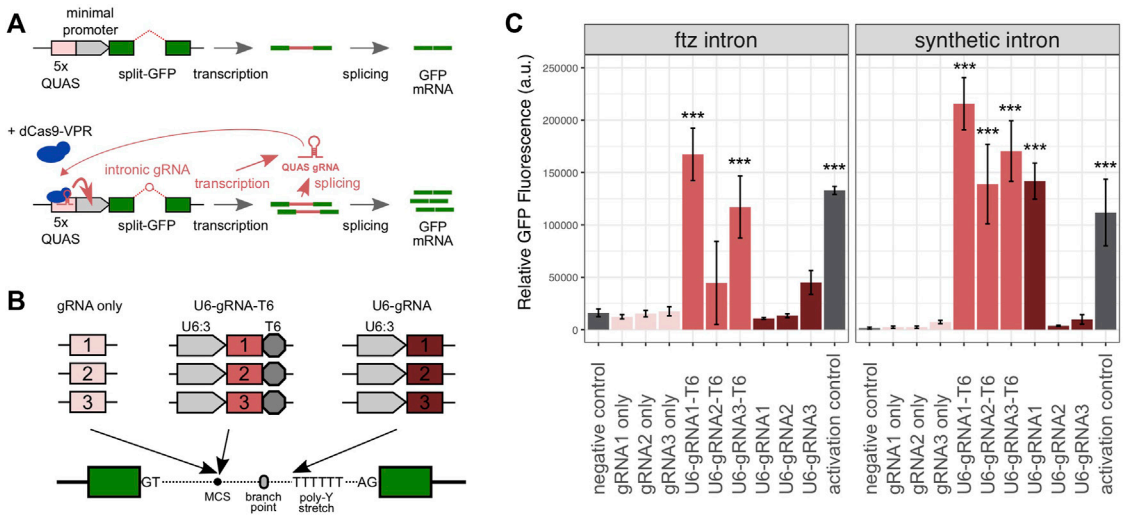

FIGURE 1 | Characterization of intron-encoded gRNAs in *Drosophila* S2 cells. **(A)** Assay consists of a GFP reporter split by the presence of an intron and the dCas9-VPR activator component. In the absence of a gRNA guiding the activator, the reporter exhibits low levels of eGFP fluorescence. Following the activation by the dCas9:gRNA complex at the 5′ QUAS motif, eGFP fluorescence is amplified. This requires both successful splicing and the gRNA expression from within the intron. Relative fluorescence is subsequently measured by flow cytometry, allowing each gRNA configuration to be compared to a negative control (no gRNA) and a positive control (gRNA provided separately). **(B)** Three intronic gRNA designs were tested with or without a U6 promoter, each with three previously characterized QUAS-targeting gRNA spacers, cloned at the multiple cloning site (MCS) near the intronic branch point, or directly adjacent to the polypyrimidine stretch toward the 3′ end of each intron. **(C)** Relative mean eGFP fluorescence and SD from triplicate transfections of each intronic gRNA variant, using either the *ftz* or minimal introns. The *p* values were calculated using one-way ANOVA and Tukey multiple comparisons of means (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

the negative controls (**Figure 1C**). The positive control yielded a strong fluorescent signal relative to the negative control (8.3-fold and 71.3-fold induction in the *ftz* and synthetic constructs, respectively), suggesting that transactivation was successful and that the intron control constructs lacking gRNAs were splicing efficiently. When analyzing combinations of gRNAs and the two intron variants, we found that no significant fluorescent induction was triggered by the constructs containing each gRNA alone (**Figure 1C**). This suggested that either splicing or the level of gRNAs liberated as spliced RNA was insufficient in these constructs. When we supplied gRNA1 for transactivation by co-transfecting the activation control plasmid, the *ftz* and synthetic minimal constructs carrying gRNA1 did yield a fluorescent signal comparable to that of the respective U6-gRNA1-T6 constructs (**Supplementary Figure S1**). This suggested that the minimal constructs did not express sufficient gRNA for sufficient transactivation but could splice efficiently when the gRNA was provided in trans. The constructs featuring the U6 promoter and terminator sequences consistently performed best in this assay for both intron types. The U6-gRNA1-T6 construct yielded a fluorescent signal 10.4-fold (*ftz* intron) and 137.5-fold (synthetic intron) higher than the controls. The U6-gRNA1-T6 construct was also the only construct performing significantly better than the activation control (1.9-fold and $p < 0.0001$), although several constructs yielded an overall higher mean level of fluorescence. Only a single experiment (U6-gRNA1 in conjunction with the synthetic intron) featuring gRNA-encoded upstream of the poly-pyrimidine stretch showed a significant level of induction (**Figure 1C**). Overall, we concluded that full gRNA expression cassettes can be located within small intron sequences, allowing both efficient gRNA expressions and splicing, and we took these designs forward for evaluation in transgenic flies.

Generation of Integral Gene Drive Strains

For the generation of autonomous integral gene drives, we chose two *Drosophila melanogaster* target loci, *bam* and *rcd-1r*, the regulatory regions of which had previously been utilized for the successful generation of gene drives in the fly (Chan et al., 2011; Chan et al., 2013; Simoni et al., 2014). These two genes were also chosen because in both cases, we were able to identify Cas9-targetable sites and PAM motif close to the start codons that would allow the insertion of the Cas9 coding sequence upstream of the coding sequence of these genes (**Figure 2A**). *Bam* (CG10422) has been associated with the fusome, a germ cell–specific organelle, and it contributes to the fate determination of germline stem cells in males and females with loss of function, leading to the production of aberrant sperm and eggs. The *bam* promoter has been frequently used to drive a germline-specific expression in *D. melanogaster*. *Rcd-1r* (CG9573) is a retrogene (Bai et al., 2007) that has been implicated in male fertility with loss-of-function mutants found to be semi-sterile or sterile (Chen et al., 2012). It shows strong expressions in adult testes, and although *rcd-1r* 5′UTR has been assayed mainly for inducing male-specific gene drives, *rcd-1r* itself is also expressed during early embryogenesis, specifically in zygotic germ cell nuclei starting from stage 8 and in early developing gonads (Tomancak et al., 2002; Lecuyer et al., 2007; Tomancak et al., 2007; Hammonds et al., 2013; Wilk et al., 2016). We next designed integration constructs where intron-encoded gRNAs

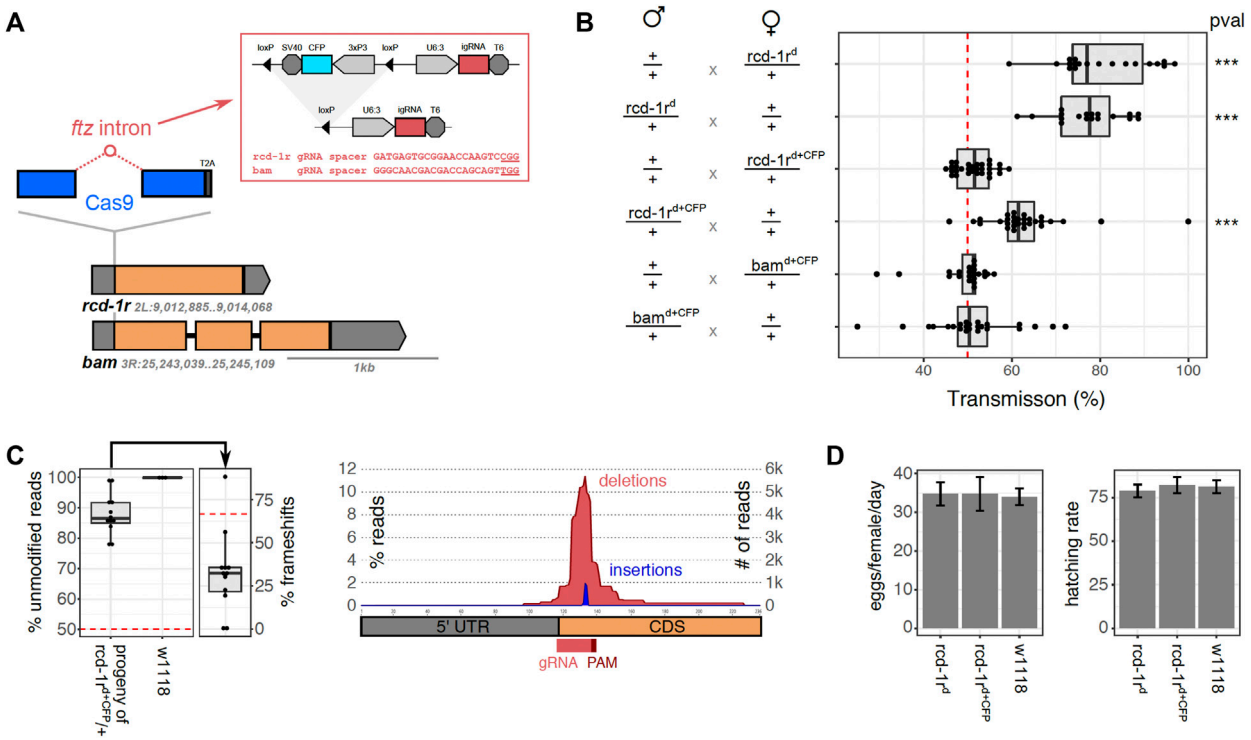

FIGURE 2 | Characterization of integral gene drive traits in transgenic flies. **(A)** Schematic representation showing the design and integration strategy of the Cas9 coding sequence (blue), hosting the *ftz* intron (red) which in turn contains the gRNA expression cassette and the excisable CFP marker cassette, at the endogenous loci *rcd-1r* and *bam* (orange) to which it is translationally linked (T2A). The gRNA target sequences (red) or the gRNA module (red circle) is indicated including the protospacer adjacent motif (underline). **(B)** Homing assays determining the rate of inheritance of the transgene to the progeny of male or female hemizygote flies. Inheritance was calculated as the percentage of the fluorescent progeny (*rcd-1r*$^{d+CFP}$, *bam*$^{d+CFP}$) or by molecular genotyping (*rcd-1r*^{d}). The *p* values were calculated using one-way ANOVA and Tukey multiple comparisons of means (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$). We performed a minimum of 18 independent crosses per condition. **(C)** Analysis of *rcd-1r* target site amplicons from the pooled progeny of *rcd-1r*$^{d+CFP}$ hemizygote males (n = 12) and a *w1118* wild-type control (n = 3). The left panel shows the median number of modified reads and the number of target site variants that are predicted to cause a frameshift. The right panel shows the size distribution of modified positions around the gRNA target site. **(D)** Analysis of fertility and fecundity of gene drive flies. The left panel shows an analysis of oviposition for homozygous transgenic strains *rcd-1rd + CFP*, *rcd-1r*^{d} and control flies performed in at least 10 independent crosses. The mean and the standard error of the mean for the number of eggs laid per female, per day (left panel), as well as the rate of eggs hatching to become adults (right panel) are shown. No statistically significant difference was found amongst the strains using one-way ANOVA and Tukey multiple comparisons of means (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

targeting *bam* or *rcd-1r* were located within the Cas9 open reading frame which was designed to link to the host locus *via* a T2A signal (**Figure 2A**). We chose the *ftz* intron which is derived from *Drosophila*, and the full U6:3-gRNA-T6 design had previously shown good performance in the cell assay. The constructs also featured a removable 3xP3-CFP transformation marker flanked by loxP sites and 5′ and 3′ regions of homology to the *bam* or *rcd-1r* loci. In both cases, the transgenes were successfully integrated into these two germline loci, which were confirmed by PCR genotyping of G1 individuals which were outcrossed to balancer strains. Following intercrosses, we observed that homozygous *bam*$^{d+CFP}$ male and female individuals were sterile, whereas we managed to successfully establish a homozygous strain *rcd-1r*$^{d+CFP}$ which showed no obvious fertility defects. In order to remove the 1.6 kb transformation marker cassette flanked by loxP sites which we expected to interfere with correct splicing and host gene expression, we next crossed homozygous *rcd-1r*$^{d+CFP}$ or TM6B-balanced bam^{d+CFP} heterozygotes to a Cre-recombinase expression strain. The transhemizygote progeny was intercrossed, and their CFP-negative progeny was then propagated in single crosses, and their offspring in turn was screened molecularly for successful excision of the fluorescent marker cassette. Following this strategy, we successfully established a homozygous strain *rcd-1r*^{d}. In the case of *bam* insertion, however, we did not recover any offspring from 10 independent single crosses of markerless individuals. This suggests that although the transgene (now consisting of only the Cas9 coding sequence and the intron-encoded gRNA) was designed to not interfere with the function of the host gene, the presence of the insert did not allow for the sufficient or correct expression of *bam* and was causing sterility.

Assaying Super-Mendelian Inheritance of the Transgenic Strains

To investigate the gene drive of the constructs established at the two loci, we conducted a series of homing assays (**Figure 2B**). We crossed male or virgin female *rcd-1r*^{d} hemizygotes, *rcd-1r*$^{d+CFP}$

hemizygotes, and *bam*$^{d+CFP}$ hemizygotes with wild-type (w^{1118}) flies and determined the transmission of the transgene in the progeny. This was either carried out by tracking the fluorescent CFP marker or, in the case of the markerless *rcd-1r*^{d} insertion, by making use of molecular genotyping. Between 18 and 30, single crosses of one male and three females were established for each condition, and crosses that failed to produce offspring were discarded. **Figure 2B** summarizes the results of these experiments. We found no significant deviation from Mendelian transmission rates for the *bam*$^{d+CFP}$ transgene (51.3 and 49.2% transgenic progeny for male and female crosses, respectively). This suggests that Cas9 and/or the gRNA were not correctly expressed in these constructs in all likelihood due to the presence of the CFP marker. Given these results and due to the observed sterility of homozygotes, for the remaining experiments, we focused on the *rcd-1r* insertions alone. We did observe a significant gene drive in *rcd-1r*$^{d+CFP}$ male (62.8% transgenics, $p < 0.001$) but not in the female (51.4%) cross. High rates of transmission were observed for the *rcd-1r*^{d} construct from both hemizygous males (77.1%, $p < 0.001$) and females (80.5%, $p < 0.001$). We concluded that although some levels of the expression of Cas9 occur in the presence of the CFP marker gene in *rcd-1r*^{d} males, more substantial rates of homing were enabled only once the marker cassette had been removed. The rcd-1r gene had previously been used for a male-specific gene drive; here however, we observed the drive in both sexes. Together with the established expression pattern of *rcd-1r* in early embryogenesis, this suggested that the observed gene drive could also be zygotic. To distinguish between the gene drive in the germline and zygotic gene drive, we crossed homozygous *rcd-1r*^{d}/*rcd-1r*^{d} males or females with the wild type. In the progeny, we did not find any evidence of somatic homozygosity in 92 genotyped individuals, all of which were *rcd-1r*^{d}/+ heterozygotes, suggesting thus that the *rcd-1r*^{d} gene drive is confined to the germline.

Analysis of Target Site Variants

In order to examine target site modifications in individuals who failed to inherit the drive construct, wild-type progenies were taken from the *rcd-1r*^{d,CFP} homing cross in pools of 50 individuals. Because we outcrossed transgenic males with wild-type females, this experiment allowed us to understand the variety of indels formed in the absence of selection to retain *rcd-1r* function. Wild-type sized alleles were amplified from a subset of these crosses (n = 12) and compared against pools of w^{1118} individuals that represent the pre-existing variation at the *rcd-1r* locus in the laboratory colony (n = 3). Resulting reads were analyzed using CRISPResso2 (Clement et al., 2019), and the indel formation rate was determined (**Figure 2C**). Reads were pooled across all biological replicates and analyzed for frequency of substitutions, deletions, and insertions proximal to the *rcd-1r* target site. We found no strong evidence of pre-existing target site variations at the *rdc-1r* locus in the w^{1118} background, but 12.1% of reads from the homing cross were found to carry indels. We found that the majority of indels were predicted to affect the rcd1-r coding sequence, but interestingly, less than the predicted 2/3 of indel events were predicted to result in a translational frameshift (32.4%). We attributed this discrepancy to the presence of a common indel variant found in 7/12 replicates and representing a total of 11% of all detected variant reads. It is likely generated by an ATGAGT microhomology with a predicted MMHEJ score of 240.8 (Bae et al., 2014) and results in a 24-bp deletion which, although it maintains the *rcd-1r* reading frame, leads to the elimination of the original start codon and its context (**Supplementary Table S1**).

Analysis of *rcd-1r*^{d} Fertility

In order to determine whether insertion of the IGD drive component affected the fitness of the transgenic *rcd-1r* strains, phenotypic assays were performed to measure basic life history traits related to fertility and fecundity. We quantified the egg output and subsequent hatching rates of homozygous *rcd-1r*^{d} and *rcd-1r*^{d,CFP} strains and the w^{1118} controls. Individual homozygote females and males were crossed in vials and allowed to mate for 24 h. At least eight replicates were performed per strain, with assays performed over 72 h, flipping mated females into a fresh vial every 24 h. The egg output was quantified per female, per day for each transgenic strain (**Figure 2D**). The vials were maintained at 25°C in order to quantify the rate of eclosion. Three days after the eclosion of pupae started, the number of adults in each vial was counted, and the hatching rate was calculated (**Figure 2D**). No statistically significant difference was observed between the *rcd-1r*^{d} and *rcd-1r*^{d,CFP} drive lines, when compared to a w^{1118} control line in terms of fertility or hatching rate. Given that *rcd-1r* had previously been implicated in male fertility, this suggested that at this locus, the IDG components, even in the presence of the full fluorescent marker cassette, did not interfere with fly fertility.

Population Cage Experiments

We next performed population cage experiments to determine the dynamic behavior of the *rcd-1r*^{d} integral gene drive in caged *Drosophila* populations of 200 individuals over 10 fly generations. We performed this experiment in four replicate populations and two release conditions where hemizygous *rcd-1r*^{d} males represented 15% or 25% of the starting male populations. This corresponds to 7.5 and 12.5% starting allele frequencies of the *rcd-1r*^{d} drive allele, respectively. Because *rcd-1r*^{d} is a markerless gene drive, we used molecular genotyping to determine the presence of hemizygous and homozygous transgenic individuals at generations 1, 3, 5, 7, and 10. We compared the observed dynamic to that predicted by a stochastic agent-based model which was parameterized with the fitness and homing rates for *rcd-1r*^{d}. We found that as predicted by the model which assumes that maintaining the *rcd-1r* function is necessary for male but not female fertility, the *rcd-1r*^{d} drive spreads rapidly but reaches a maximum allele frequency of approximately 80% with a similar peak frequency under both release conditions. These frequencies are slightly less than those predicted by the models and could be due to the effects that we did not account for computationally, for example, low levels of maternal Cas9 deposition into the embryo. This experiment validates the results of the individual genetic crosses and shows a successful drive of a minimal integral gene drive at the population level. We performed Sanger sequencing of non-transgenic individuals to

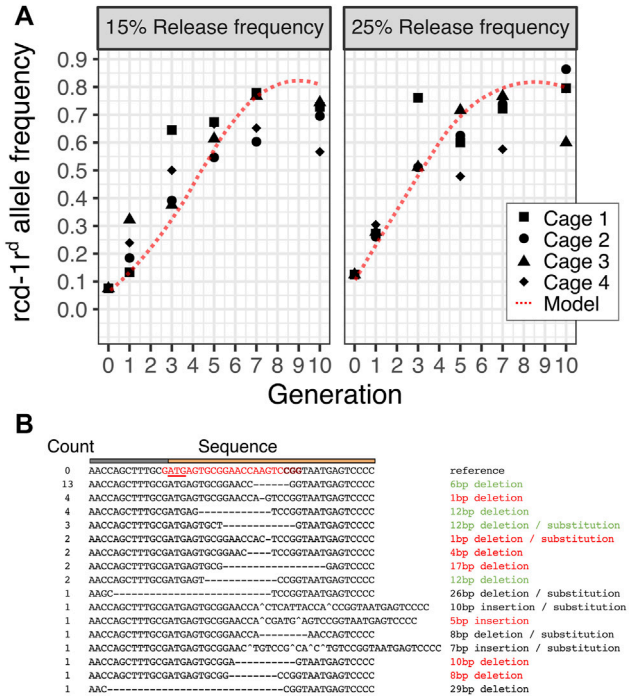

FIGURE 3 | Population dynamics of the *rcd-1r*^{d} gene drive. **(A)** The predicted allele frequency of *rcd-1r*^{d} in four replicate cages maintained for 10 generations and sampled at generations 1,3,5,7, and 10 at two initial release frequencies (15% or 25% hemizygote *rcd-1r*^{d} corresponds to a starting allele frequency of 7.5% or 12.5%, respectively) is shown. The dotted red line represents the predicted frequency according to the mean of 25 runs of a stochastic model of the *rcd-1r*^{d} drive. A minimum of 31 and a mean of 42 individuals were genotyped per condition, cage, and generation. **(B)**. Summary of target site variants identified by Sanger sequencing in generation 10 using hemizygous individuals from all eight cage populations. The putative impact of the mutation as disruptive (red), possibly tolerable (green), and ambiguous/unclear (black) is indicated.

determine target site variants that had accumulated in these cage populations by generation 10. Out of a total of 40 samples, 13 carried a 6-bp deletion which was detected in six out of the eight cage populations (**Figure 3B**, **Supplementary Table S1**). This suggests that selection for maintaining the *rcd-1r* function leads to the rise of a prominent variant featuring a two-amino acid deletion at the protein's N-terminus.

DISCUSSION

Here, we sought to generate an autonomous gene drive consisting of a minimum number of genetic components in *Drosophila melanogaster*. Our aim was to design a neutral gene drive that would seed a population with a source of Cas9 but cause no other intended phenotypes. We first characterized, in cell culture experiments, intron modules that allowed both intron splicing and the U6-driven expression of the intron-encoded gRNAs. Although promoterless intron variants were also tested and the liberation of spliced introns as gRNAs had previously shown to be attainable, such variants failed to achieve high levels of activity in our assay. We next showed that U6-driven gRNA-expressing introns, when embedded within the Cas9 open reading frame, could constitute a functional gene drive in transgenic flies. Such a module, when integrated at the N-terminus of the germline-expressed *rcd-1r* gene and translationally linked to its expression, showed high levels of the gene drive in the male and female germline, while at the same time we could not detect any negative effects on fly fertility. This approach was not as successful at the *bam* locus where the introduced sequences evidently interfered with the host gene expression, leading to sterility. The intronic gRNAs we described could have other applications besides the gene drive. For example, intronic gRNAs could be integrated at or around rare polymorphisms of genes of interest as they would be neutral with regards to the host gene function. If such gRNAs target the wild-type allele, they could, when paired with a source of Cas9, drive the allelic conversion of a tissue or a population. Such a strategy could be used to study the function of rare alleles.

We studied the behavior of the *rcd-1r* gene drive at the population level and found that the spread of *rcd-1r*^{d} matched the dynamic predicted by a simple discrete-generation model. The model assumes that the loss of the *rcd-1r* function (R2 homozygote genotype) leads to a 95% reduction in male fertility, and thus, these R1 alleles will be selected. When we analyzed the generation of resistant alleles, we found that in single crosses, in the absence of selection, there was a preferred repair outcome which was likely the result of microhomology around the *rcd-1r* gRNA target site. This variant, however, was not detected at all in the cage populations where the most common variant was found to carry a two-codon deletion, which was likely selected for its ability to maintain *rcd-1r* function. Markerless gene drives, as described herein, can only be detected by molecular methods. When the target site and genomic location of the gene drive is unknown and when, as we show, there is a dearth of additional exogenous sequence elements, genotyping would likely require targeting the Cas9 ORF itself. Due to the possibility of recoding of the ORF (or the use of alternative CRISPR endonucleases) and the presence of introns, it is possible that the standard detection method could easily be circumvented. Although we used a conventional Cas9 ORF, the presence of the gRNA intron would cause standard Cas9 primers to not yield the correct product for the *rcd-1r*^{d} gene drive. In similar designs, only whole genome sequencing or protein-based detection methods could reliably identify gene drive individuals. These considerations should inform discussions around the biosecurity of the gene drive as it is possible to design minimal and recoded gene drives that could propagate in populations undetected (e.g., if a deliberate or accidental release of an unregulated or "garage" gene drive were to occur). Our results thus demonstrated that the generation of autonomous gene drives with a minimum number of genetic components is achievable (consisting of only a Cas9 coding sequence hosting an intronic gRNA). Further experiments, including experiments in non-model target organisms such as malaria mosquitoes, will be required to see if this approach is more broadly applicable. In particular, the targeting of genes with a more conserved N-terminus and males and females could avoid some of the issues encountered at the *rcd-1r* locus. Targeting

highly conserved sequences has been shown to alleviate some of the problems related to the formation of resistant alleles (Kyrou et al., 2018); however, the N-terminal may offer fewer such conserved targetable sites. We have already used the methods described herein to develop analogous non-autonomous gene drives in the malaria mosquito (Hoermann et al., 2021). These traits expressed antimalarial effectors from within mosquito host genes and carried gRNA-expressing introns based on the designs we described here. When combined with Cas9 expressor strains, autonomous and non-autonomous gene drives could constitute a modular system to test gene drives and their effector mechanisms at an increasing scale moving from the laboratory to the field.

MATERIALS AND METHODS

Cell Culture and Transfection

Experiments were performed in Schneider 2 cells (Thermo Fisher Scientific, United Kingdom). Cells were cultured at 25°C in an ambient atmosphere. Cells were maintained in a complete growth medium composed from 90% Schneider's *Drosophila* medium (Thermo Fisher Scientific, United Kingdom), and 10% FBS (Sigma-Aldrich, United Kingdom). Cells were maintained in T-25 flasks, with 0.2-μM filter caps. Visualization was performed using a Nikon TMS inverted microscope and a ×10 confocal lens. Cells were passaged biweekly, at 1:4 ratios, at which point they had achieved a density of $>1 \times 10^6$ cells/ml. The cell count was performed using a Scepter 2.0 Cell Counter. Confirmation of the mycoplasma-free status was performed by PCR. Constructs used for experimental work in S2 cells were prepared for transfection by being midiprepped using *E. coli* TOP10 cells. Following transformation, individual colonies were selected and used to inoculate 5 ml of LB-Miller starter cultures, supplemented with ampicillin. Starter cultures were incubated for 8 h at 37°C in a shaking incubator at 220 rpm. They were then used to inoculate 500-ml falcon flasks containing 100 ml of LB-Miller broth, supplemented with ampicillin. Falcon flasks were incubated overnight at 37°C in a shaking incubator at 220 rpm.

DNA Constructs

Annotated DNA plasmid sequences have been provided in **Supplementary Material S1**. Briefly, sequences for the constructs used for the cell-based assay were derived from pQUAST (Addgene plasmid #24349) by the insertion of gene-synthesized fragments and validated by Sanger sequencing. To generate U6:3-gRNA-T6 gRNA cassettes for the generation of gene drive constructs, spacers (*bam GTCGGGCAACGACGACCA GCAGT*, *AAACACTGCTGGTCGTCGTTGCCC*; and *rcd-1r GTC GATGAGTGCGGAACCAAGTC*, *AAACGACTTGGTTCCGCA CTCAT*) were synthesized as two complementary ssDNA oligos (Eurofins Genomics, Ebersberg, Germany), with BbsI compatible overhang sites in the pCFD3-dU6:3gRNA (Addgene plasmid #49410) (Port et al., 2014), which were then cloned into the Bsp119I site within pBS-Hsp70-SpCas9-ftz, followed by the subsequent insertion of the 3xP3:CFP:SV40 cassette. For each locus, 1-kb homology arms were synthesized (ATUM, CA, United States), and BbsI or BaeI sites were introduced to produce a linearized backbone into which the drive cassette was inserted by the Gibson assembly.

Flow Cytometry

Fluorescence in S2 cells was analyzed by using a three-laser BD LSRFortessa analyzer. Live, single cells were selected by gating forward and side scatter. For each condition analyzed, at least 500 gated events were recorded, with three replicates per condition. The following voltages were used—forward scatter (FSC): 170 V, side scatter (SSC): 253 V, 530-nm channel: 390 V, and 610-nm channel: 591 V. Excitation of eGFP was performed using a 488-nm blue laser and by using a 530/30 bandpass filter. The excitation of mCherry was performed using a 561-nm yellow laser and by using a 610/20 bandpass filter. Transfection of single color constructs was used for the construction of compensation matrices to account for spectral overlap.

Transgenic Flies

Embryos were dechorionated in 50% bleach, prior to microinjection and aligned on 10 × 10 mm coverslips. The needles were pulled using a P1000 Sutter micropipette puller (Sutter, United Kingdom). Injections were conducted with w^{1118} embryos at 500 ng/μl, consisting of a 400 ng/μl drive construct and 100 ng/μl pnos-Cas9 helper plasmid. Following microinjection, embryos were retained on coverslips and placed onto 100 mm × 100 mm agar plates. Upon hatching, first instar larvae were placed into a yeast paste and transferred to plugged vials to mature at 22°C. All stocks were maintained at 18°C, while all experiments were conducted at 25°C under Arthropod Containment Guidelines Level 2 conditions.

Cre-Mediated Reporter Excision

Rcd-1r drive lines were generated by performing 10 single crosses of $rcd\text{-}1r^{\mathrm{d,CFP}}$ with *y1* w^{67c23}; sna^{Sco}/*CyO*, and Pw [+mC] = CrewDH1 virgins to excise the 3xP3:CFP:SV40 reporter cassette located within the Cas9-located *ftz* intron of the drive component. G1 progenies were all markerless and were intercrossed to produce homozygous $rcd\text{-}1r^{d}$ G2 progenies. Excision of the CFP reporter cassette was confirmed by Sanger sequencing.

Fertility Assay

The oviposition and hatching assay (Simoni et al., 2014) was performed using the w^{1118} line as a control. Virgin females and males were collected and aged for 24 h. Single crosses were subsequently performed, with mating being allowed to proceed for 48 h. Males were subsequently removed, and females were allowed to lay eggs in vials containing standard yeast food for three consecutive days, with flies transferred into a new vial every 24 h. At least eight replicates were performed per line, with the number of eggs per female per day quantified. The number of eclosed adults arising from said eggs was then quantified, with at least eight replicates observed per line.

Homing Assay

Hemizygotes were obtained by crossing single *rcd-1r* and $rcd\text{-}1r^{\mathrm{d,CFP}}$ males to w^{1118} virgins. In the case of *bam* balanced w^{1118}, *bam*/TM6B and Tb [1] males were crossed with w^{1118} virgins. Subsequent G1 heterozygotic progenies were screened such that only w^{1118} *bam*

individuals were selected to act as parents for homing assay crosses. Hemizygous progenies were collected such that the females were virgins, and all individuals were 1–3 days old. Single males were crossed with three females in each cross. Male and female hemizygous transgenics were assayed separately to determine their sex-specific effects. In cases where replicates failed to yield offspring, they were discarded and where lines carried a CFP reporter cassette, the gene drive carrying the progeny was identified using an Olympus MVX10 fluorescent microscope for fluorescence. A markerless *rcd-1r* progeny was genotyped by extracting genomic DNA using Chelex 100 resin. An extraction solution was produced by suspending 1.25 g of Chelex resin in 100 ml of RNase-free water supplemented with 4 ml of proteinase K solution (20 mg/ml). This solution was added in 100 µl aliquots to each well containing a fly. Plates were sealed using adhesive plate foils. Incubation occurred in an Eppendorf Thermomixer-C, for 2 h at 55°C and 700 rpm, and then, proteinase K was inactivated by incubation for 20 min at 99°C. Plates were stored at −20°C. PCR reactions using primers TAGCAAAGTCAGGGCGTAGC and CACCGGGATAAGCCCATCAG using REDTaq DNA polymerase were conducted as follows: 98°C for 2 min, followed by 35 cycles of 98°C denaturation step for 20 s, a 59°C annealing step for 30 s, and a 72°C extension step for 30 s, followed by a final 72°C extension step for 7 min, and then a 4°C hold. PCR reactions were visualized using 1% agarose gels, and the presence of a 609-bp band was considered to be a positive indication of a transgenic individual.

Amplicon Sequencing

Amplicon sequencing was performed on a CFP-negative progeny of the *rcd-1r*^{d,CFP} homing assay. In total, 50 CFP-negative G2 progenies were selected at random from a given biological replicate, and pooled genomic DNA extraction was performed for 12 replicates. Then, 200 control *w*^{1118} flies of a commensurate age were selected, and pooled genomic DNA extraction was performed in three replicates. Qubit quantified yields were normalized to 50 ng/µl using Tris-EDTA. PCR was then performed using primers ACACTCTTTCCCTACACG ACGCTCTTCCGATCTGACGCTTTTCCACAGCATGG and GACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTCGGTC CTTTCTCGCTTGA producing an amplicon of or around 320 bp. Primers included a partial scaffold compatible with Illumina NGS sequencing. PCR conditions were as follows: 98°C denaturation step for 2 min, followed by 23 cycles of 98°C for 10 s, 57°C for 10 s, and 72°C for 30 s, followed by a final 72°C extension for 7 min, and a 4°C hold. Successful amplification of PCR products was confirmed by loading 5 µl of each sample onto a 1% agarose gel, running at 100 V for 60 min, and visualizing. PCR products were subsequently purified using a QIAGEN MinElute PCR Purification kit (QIAGEN, CA, United States), and purified DNA products were eluted in 10 µl Tris-EDTA. Purified DNA was quantified using a Qubit fluorometer as before and normalized to 20 ng/µl, in at least 20 µl of the total volume using Tris-EDTA. Samples were processed by Genewiz (Genewiz, Leipzig, Germany), and the data were processed using CRISPResso2 (Clement et al., 2019).

Population Cage Experiments

Experiments were performed in a 250-ml conical bottle, plugged with cellulose acetate plugs (VWR, United Kingdom) and filled with 50 ml of standard yeast-based fly food (12lt water, 240 gr polenta, 90 gr agar, 960 gr fructose, 1,200 gr brewer's yeast, 60 ml Nipagin 15% w/v in ethanol, and 90 ml propionic acid). Flies were allowed to mate for 5 days, before being recovered using CO_2, and stored at −80°C for subsequent analysis. Three days after G1 individuals had eclosed, the adults were retrieved from each bottle and counted. Then, 200 individuals were selected at random and placed into a fresh bottle to begin the next generation. Any excess flies were frozen at −80°C for later analysis. This cycle was repeated until generation 10. To genotype DNA, extraction was performed as described, and multiplex PCR reactions were setup in 96-well plates, corresponding to each gDNA plate. Primers AGAAGCTGTCGTCCACCTTG, ACG TGCTTTCGGTCCTTTCT, and AGGTGTTCTTGCTCAGCT CC that bind to the transgene and the endogenous locus generate a product of 586 bp when the *rcd-1r*^{d} allele is present and a 292-bp product for the wild-type allele. We used the REDTaq DNA polymerase mastermix (VWR, United Kingdom) as follows: denaturation at 98°C for 2 min, followed by 35 cycles of the 98°C denaturation step for 20 s, 59°C annealing step for 30 s, and the 72°C extension step for 40 s, followed by a final 72°C extension step for 7 min, and then a 4°C hold. Plates were sealed, as before, with an adhesive foil. A total of 10 *rcd-1r*^{d} hemizygous G10 samples from each replica of both release conditions were further analyzed through Sanger sequencing in order to determine the presence of target site variants.

Gene Drive Model

We employed a simple object-oriented stochastic agent-based discrete generation model written in C#. The model allowed to fully account for the genetic interactions of autonomous and non-autonomous CRISPR-based drive elements and the generation and tracking of R1 and R2 resistance alleles at all modeled loci. Briefly, we considered populations of 200 individuals where WT, R1, R2, and drive constituted the possible genotypes at the *rcd-1r* locus. We included the experimentally observed drive parameters and a 95% loss of male fertility for a loss of the *rcd-1r* function (R2 homozygotes). In each generation, all adults were randomly mated, and 200 offspring were randomly selected for constituting the next generation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AN and NW designed the study. AN, PC, AH and PP performed experiments. AN, PC, AH, PP and NW analyzed the data. NW and AH wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.857460/full#supplementary-material

Supplementary Figure S1 | Testing intronic gRNA designs in the presence of the activation control to determine splicing levels. Relative mean eGFP fluorescence and SD from triplicate transfections of each intronic gRNA variant, using either *ftz* or minimal introns. The *p* values were calculated using one-way ANOVA and Tukey multiple comparisons of means (*p<0.05; **p<0.01; and ***p<0.001).

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Impact of Irradiation on Vector Competence of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) for Dengue and Chikungunya Viruses

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Effective control strategies against arthropod disease vectors are amongst the most powerful tools to prevent the spread of vector-borne diseases. The sterile insect technique (SIT) is an effective and sustainable autocidal control method that has recently shown effective population suppression against different *Aedes* vector species worldwide. The SIT approach for mosquito vectors requires the release of radio-sterilized male mosquitoes only, but currently available sex separation techniques cannot ensure the complete elimination of females resulting in short-term risk of increased biting rate and arboviral disease transmission. In this study, we compared for the first time the transmission of dengue and chikungunya viruses in *Aedes aegypti* and *Aedes albopictus* females exposed as pupae to an irradiation dose of 40 Gy. Females of both species were fed on blood spiked with either dengue or chikungunya viruses, and body parts were tested for virus presence by real-time RT-PCR at different time points. No differences were observed in the dissemination efficiency of the dengue virus in irradiated and unirradiated *Ae. albopictus* and *Ae. aegypti* mosquitoes. The dissemination of the chikungunya virus was higher in *Ae. albopictus* than in *Ae. Aegypti*, and irradiation increased the virus load in both species. However, we did not observe differences in the transmission efficiency for chikungunya (100%) and dengue (8–27%) between mosquito species, and irradiation did not impact transmissibility. Further implications of these results on the epidemiology of vector-borne diseases in the field are discussed.

Keywords: sterile insect technique, dissemination, transmission, arbovirus, RRT-PCR

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INTRODUCTION

Despite control measures applied worldwide over decades, mosquito-borne diseases continue to pose a constant threat to human and animal health. Globalization and climate change are resulting in the increased movement of mosquitoes and introductions and establishment of mosquito populations in areas where they could not survive before. This has contributed to a resurgence of important known diseases such as dengue fever, caused by the dengue virus (DENV), and new viruses which only

recently have demonstrated their enormous pathogenic potential, such as the virus responsible for the Zika disease (ZIKV). *Aedes albopictus* and *Aedes aegypti* are considered the two most important mosquito vectors responsible for transmitting dangerous arboviruses circulating in tropical and temperate areas and that have the potential of imposing a significant global disease burden on half of the world's population. Mosquitoes are recognized as the most invasive and deadly animal species in the world, and many of these species have expanded their distribution in all continents primarily through human-mediated transportation, despite attempts to reduce their density and prevent their establishment (Benedict et al., 2007; Paupy et al., 2009; Medlock et al., 2012; Schaffner et al., 2013; Schaffner et al., 2014). The increased and rapid development of the resistance of mosquitoes against existing and newly developed insecticides, the paucity of specific drugs or the lack of the development of new drugs, and the absence of effective vaccines against most of these arboviruses have stimulated the evaluation of alternative, sustainable, and effective mosquito control methods to successfully reduce the density and distribution of these important sanitary pests (Medlock et al., 2012).

The application of effective vector control tactics remains the main strategy for the management of many vector-borne diseases and the only approach available to protect populations against this nuisance (Wilson et al., 2020). The sterile insect technique (SIT) is an autocidal pest control method that requires the area-wide inundative releases of sterile insects (FAO, 2005) to induce sterility in the native female pest population. Consequently, the reproduction rate of the target population declines, resulting in a reduced density of the field population with each generation. It is, therefore, a type of "birth control" as wild female insects of the pest population do not reproduce when they are inseminated by the released sterilized males. In this type of autocidal control, sequential releases of sterilized insects in adequate sterile to wild male overflooding ratios lead to vector suppression, and hence, to the containment of the diseases these vectors transmit (Dame et al., 2009; IAEA et al., 2012; Bellini et al., 2013; Dyck et al., 2021). The SIT has been used all over the world as a part of area-wide integrated pest management (AW-IPM) programs over the past 70 years to successfully contain, reduce, eliminate, or prevent the establishment of insect pests of agricultural, veterinary, and medical importance (Dyck et al., 2021). In the last decade, several SIT pilot field trials have been implemented against several *Aedes* vector species worldwide with promising results (Oliva et al., 2021). However, many improvements in the mosquito "SIT package" are required to advance this control tactic toward a larger-scale operational level (Bouyer et al., 2020a; WHO/IAEA, 2020; Oliva et al., 2021). Sex separation remains one of the main challenges for the efficient application of the mosquito SIT. The release of sterile female mosquitoes has to be avoided at all costs as they could contribute to the transmission of these viruses. Although mechanical sex separation techniques of *Aedes* pupae do exist, a small percentage of females is still accidentally processed together with the males and then released in the field. It is not expected that the release of a small percentage of unwanted sterile females together with the males would have a significant impact on the efficiency of the SIT, but it might increase the biting rate, and hence, the risk of transmission of these arboviruses which will limit its applicability and more importantly, its political and ethical acceptability, especially in areas where these diseases are endemic (WHO/IAEA, 2020).

Virus transmission by arthropods is a complex process (vector competence) controlled by biological intrinsic barriers in the vectors, including barriers affecting virus amplification in the mid-gut cells, dissemination in the insect's body fluid (hemolymph), and finally, infection and amplification in the salivary glands as the last stage before transmission to a new host. Ionizing radiation can influence the competence of different mosquitoes for pathogens and viruses through alterations of their immune response, and therefore, their ability to effectively transmit these arboviruses (Guissou et al., 2020). Radiation can also seriously alter symbiotic gut microbiota which is linked to the insect ecological fitness (Cai et al., 2018) and can affect immune responses that can influence vector competence for human pathogens (Dennison et al., 2014). Moreover, radiation can alter the feeding behavior and the survival rate of females under laboratory conditions (Cunningham et al., 2020). All of the aforementioned suggests the need for a more in-depth investigation of the effect of irradiation on vector competence in *Aedes* female mosquitoes.

The present study is the initial step of a more comprehensive investigation of the effect of irradiation (40 Gy) administered to *Ae. albopictus* and *Ae. aegypti* female pupae, on the transmission of dengue (DENV) and chikungunya viruses (CHIKV). We measure the viral RNA load of DENV and CHIKV in the saliva of irradiated and nonirradiated adult female mosquitoes as a means of transmission efficiency and transmission rate. Saliva samples were analyzed as individual females and in pools of eight samples/pool to compare the sensitivity of the test. Moreover, viral RNA load was also investigated in other body parts, to evaluate the propagation of the two viruses (dissemination efficiency) within the two species of irradiated and nonirradiated mosquitoes. The results will contribute to a better understanding of the implications and risks of unintentionally released irradiated *Aedes* females during AW-IPM programs that include an SIT component.

MATERIALS AND METHODS

Mosquito Strains

The *Ae. albopictus* and *Ae. aegypti* strains used in this study were obtained from eggs collected in Rimini (Emilia-Romagna, Italy) and Juazeiro (Bahia, Brazil), respectively, by the Centro Agricoltura Ambiente CAA Italy and Moscamed Brasil, both IAEA collaborating centers for the development of the SIT package against *Aedes* vectors. Eggs were received from the FAO/IAEA Insect Pest Control Laboratory (IPCL, Seibersdorf, Austria) and reared under laboratory conditions (27 ± 1°C, 85 ± 5% RH, 16:8 h L:D photoperiod) at the National Centre for Vector Entomology of the University of Zürich (UZH, Zürich, Switzerland). Larvae obtained after standardized hatching procedures (Balestrino et al., 2010) were reared at a fixed

larval density (2 larvae/mL) and fed with IAEA-BY liquid diet (3.0% w/v) at a mean daily dose of 0.5 mg/larvae (Balestrino et al., 2014) for the first four days of development. Pupae harvested on the sixth day from larval introduction were sexed under a stereomicroscope and aged at least 24 h before being subjected to irradiation treatments.

Mosquito Irradiation

About 1,000 *Ae. albopictus* and *Ae. aegypti* female pupae were transferred into separated tissue culture flasks (type T75, 250 ml capacity) each containing 210 ml of deionized water for irradiation procedures. The quantity of water in the flask was used to standardize the pupal irradiation exposure during treatment by maintaining the floating position of the pupae at 3 cm from the flask's bottom. Flasks were transported to the Department of Radiation Oncology, University Hospital of Zürich (USZ), where irradiation was carried out with a TrueBeam linear accelerator (TrueBeam® STx, Varian Medical Systems, Palo Alto, CA) at 40 Gy (dose rate 6.2 Gy/min, photons energy 6.0 MV) [48]. Immediately after irradiation, the pupae were transported back to the UZH Insectary and placed in plastic cups (diameter 7 cm, height 8 cm) with about 150 ml of deionized water for emergence inside two separated polyester netting cages (32.5 × 32.5 × 32.5 cm) (BugDorm 43030F, MegaView Science Co., Ltd., Taichung, Taiwan). Each cage was provided with about 1,000 conspecific fertile males to assure the mating status of females and their optimal post-mating biting behavior. A 10% sucrose solution was supplied as a carbohydrate source and the cages were kept in a climate chamber under laboratory conditions (27°C with 85% RH and 16:8 h L:D photoperiod). In this preliminary study, we applied a dose of 40 Gy to 24–30 h old *Ae. aegypti* and *Ae. albopictus* pupae, shortly before adult emergence. This dose and time of treatment were selected as it resulted in male residual sterility of around 1% for both species with a low impact on sterile insect quality (Balestrino et al., 2010; Balestrino et al., 2017; Culbert et al., 2018).

Viruses

Two viruses were used for mosquito oral inoculation: dengue type 2 virus strain (Vazeille et al., 1999) (DENV-2) from Bangkok and chikungunya strain 06.21 (Vazeille et al., 2007) (CHIKV) from La Réunion Island. Both viruses were amplified twice in C6/36 cell lines before their use for oral inoculation of mosquitoes. Briefly, C6/36 cells were grown in cell culture flasks (T25, 50 ml capacity) maintained in a Leibovitz L-15 medium supplemented with 1% antibiotics-antimycotics (penicillin, streptomycin, and amphotericin B), and fetal calf serum (FCS) at a concentration of 4 and 10% for CHIKV and DENV, respectively. Confluent C6/36 cells were inoculated with 100 µL of the original virus generating a C6/36 passage 1 (P1). All the flasks were incubated for three days (CHIKV) and five days (DENV) at 28°C with 5% CO_2. After the incubation period, the supernatants were harvested and 200 µL inoculated into a new C6/36 cell flask (T75, 250 ml capacity) to generate a P2 passage at a multiplicity of infection (MOI) of 0.1. To confirm the infectivity of all passages, 10-fold serial dilutions of P1 and P2 supernatants were titrated on 96-well plates layered with C6/36 cells. Briefly, 96-well plates were seeded with 6.2×10^6 cells/mL and incubated at 28°C and 5% CO_2 for 24 h before their inoculation with the virus. For the inoculation, media were removed from all the well plates followed by inoculation of 50 µL/well of each ten-fold serial dilutions (using four replicates per dilution) and incubated for 1 h at 28°C and 5% CO_2. After the incubation time, each well was overlaid with 150 µL of a premix 1:1 of sterile CMC (carboxymethylcellulose sodium salt, Sigma), 3.2% (water and 0.85% NaCl), and an L-15 medium supplemented with 10% FCS, and then the plates were sealed and incubated at 28°C with 5% CO_2. On day three (CHIKV) or day five (DENV) post-incubation, the cells were fixed by adding 100 µL/well of formaldehyde 3.6% (in PBS) (without removal of the overlay) followed by an incubation period of 20 min at RT. After this time, the content from each well (medium and formaldehyde) was removed and the cells were rinsed three times with PBS. To detect viral foci, an immunoperoxidase assay was performed. Briefly, the wells were incubated in Triton (0.5% in PBS) for 5–15 min at RT followed by three PBS washes. The wells were incubated for 30–45 min at 37°C with the primary antibody at the appropriate concentration in PBS (1:200 for DENV and 1:1000 for CHIKV) followed by three PBS washes. The secondary antibody goat anti-mouse igG (H + L) (Alexa Fluor® 488; ThermoFisher Scientific Inc., USA) was added (1:500 in PBS for both viruses) and incubated for 30 min at 37°C and the final three PBS washes were applied before examination of the fluorescence in each well by an indirect immunofluorescence assay (IFA). Tissue culture infectious dose ($TCID_{50}$) of both viruses was calculated by the observation of fluorescence at each dilution series and the titers were calculated as the last dilution recorded positive at the 50% end-point and expressed as $\log_{10}TCID_{50}$/mL which was later converted to $\log_{10}$ plaque-forming units/mL ($\log_{10}$ PFU/mL) (O`Reilly et al., 1994). Finally, a standard curve of the two viruses was generated by converting the viral RNA amount cycle threshold (Ct) values into PFU. Briefly, viral RNA from the supernatant of the second virus passages (DENV C6/36 P2 and CHIKV C6/36 P2) was extracted using a viral nucleic acid kit (Qiagen QIAmp viral RNA mini kit), and a real-time reverse transcriptase polymerase chain reaction (rRT-PCR) of ten-serial fold dilutions of viral RNA was performed (Mousson et al., 2010; Mousson et al., 2012). Finally, Ct values from each serial dilution were challenged toward the standard curves to infer the equivalent number of PFU/mL to the Ct values.

Mosquito Infection

Infection and incubation of mosquitoes were carried out in the BSL3 laboratory of the Laboratory Animal Service Centre (LASC) at the UZH. Seven-day-old *Ae. aegypti* and *Ae. albopictus* irradiated females (F0), caged with conspecific fertile males, were deprived of sugar for 24 h before their exposure to heparinized sheep blood spiked with either CHIKV or DENV at a final concentration of 8.0 $\log_{10}TCID_{50}$/mL (17.2 Ct) and 6.0 $\log_{10}TCID_{50}$/mL (21.21 Ct), respectively. The virus-spiked blood was transferred to a Hemotek feeder (Hemotek Ltd., Lancashire, United Kingdom) layered with a membrane Parafilm M (Sigma-Aldrich, Buchs, Switzerland) and kept at a constant temperature of 36 °C during the whole feeding process. After 30 min of

feeding, fully engorged females of both species were transferred into a netted cardboard pot (approximately 80 females/pot) with a 10% sucrose solution imbibed cotton pad on top of the net. All the pots were incubated in a climate chamber as described previously for 7 (CHIKV) or 14 (DENV) days post-inoculation (dpi). As a control, unirradiated mosquitoes from both species were exposed to CHIKV or DENV, incubated, and processed as irradiated mosquitoes. Freshly engorged *Ae. aegypti* and *Ae. albopictus* females for each irradiated and unirradiated group were collected at day 0 (day 0 females) and a sample of the infectious blood mixtures used for oral inoculation and processed for rRT-PCR to confirm infection and provide baseline data.

Mosquito Dissection and Saliva Collection

After the incubation period, 100 surviving female mosquitoes from each treatment were processed. To collect saliva from live females, legs and wings were removed and stored in individual 1.5 ml Eppendorf tubes previously filled with 100 µL of DMEM supplemented with 1% antibiotics-antimycotics (penicillin, streptomycin, and amphotericin B) and 10% FCS. Females deprived of legs and wings were allocated on a flat surface and their proboscides were inserted into 5 µL glass capillary tubes filled with 10% FCS. Females were left in this position for 30 min after which the proboscides were removed from the capillary tubes and the contents of the capillary tube with the possibly spitted saliva flushed into a 1.5 ml Eppendorf tube filled with 400 µL of DMEM (10% FCS and 1% antibiotics-antimycotics as described previously). After saliva collection, the bodies of all females were dissected into 1) head and thorax and 2) abdomen, which were individually stored in separated 1.5 ml Eppendorf tubes at −80°C until further examination.

Viral RNA Quantification

Dissemination of CHIKV and DENV was investigated by quantifying viral RNA (rRT-PCR) isolated from the legs and wings. These body parts from individual females were homogenized using the TissueLyser II instrument (TL) (Qiagen GmbH, Hilden, Germany). Briefly, one stainless steel bead (3 mm diameter) was added to each tube together with 100 µL of DMEM supplemented as described previously and processed with the TL at 25 Hz for 1 min. Additional 900 µL of DMEM was then added yielding a final volume of 1.0 ml. After centrifugation at 13,000 rpm for 5 min, 100 µL aliquots from eight samples were pooled (800 µL total volume/8 females). Viral RNA was extracted from each pool using the manual viral nucleic acid extraction kit (QIAmp viral RNA mini kit) described previously and rRT-PCR was performed as described elsewhere (Mousson et al., 2010; Mousson et al., 2012). Dissemination efficiency corresponds to the proportion of mosquito pools with infected legs and wings among tested ones.

To confirm the presence of viral RNA in saliva (transmissibility), the saliva of individual females that were positive in legs and wings (pools) were pooled and processed for viral RNA detection using the protocols described previously. Briefly, viral RNA was extracted from all females (n = 16) originated from two saliva positive pools (16 females per type of treatment [irradiated *vs.* unirradiated], vector species [*Ae. albopictus vs. Ae. aegypti*], and virus [CHIKV *vs.* DENV]). Saliva pools were chosen according to their Ct values: one pool with the highest Ct values and one with the lowest. Transmission efficiency corresponds to the proportion of mosquito pools with the virus in saliva among tested ones.

Statistical Analysis

All statistical analyses were carried out using R statistical software (version 3.5.2). Pearson's Chi-square test was applied to compare the dissemination and transmission efficiency for dengue and chikungunya viruses among irradiated and unirradiated *Ae. albopictus* and *Ae. aegypti* mosquito pools. The normality of the individual and pool data distributions was assessed using the two-sample Kolmogorov–Smirnov test. The data of the measurements of viral loads were normally distributed ($p > 0.05$). Concordance between individual values and their corresponding positive pools was determined by measuring the datasets through the Pearson correlation test. The viral loads observed were about 1–3 logs lower in mosquitoes infected with DENV than with CHIKV which were thus analyzed separately. A Gaussian linear mixed-effects model fit by maximum likelihood was used to analyze the virus titers (PFU/ml) separately for the two viruses (CHIKV *and* DENV) with mosquito species, irradiation, and their first-order interaction as fixed effects and the replicate as a random effect. The best model was identified by simplifying the complete one and considering the one with the lowest corrected Akaïke information criterion. The significance of the fixed effects was tested using the likelihood ratio test to compare models with or without these effects (Hurvich and Tsai, 1995; Burnham and Anderson, 2003).

RESULTS

Mosquito Infection

Overall, 411 irradiated female *Ae. albopictus* and 427 *Ae. aegypti* were exposed to heparinized sheep blood spiked with DENV, and 296 irradiated female *Ae. albopictus* and 458 *Ae. aegypti* were exposed to CHIKV-spiked blood. The viral RNA amounts (Ct) measured in the infectious blood and in the day 0 females ranged between 25.7 and 35.0 Ct.

Viral RNA Quantification

The virus inoculum (P0) used to generate P2 were 6.8 and 9.3 Log_{10} (PFU)/mL for DENV and CHIKV, corresponding to 19.26 and 13.44 Ct values for DENV and CHIKV, respectively. Standard curves of the DENV and the CHIKV (C6/36, P2) used in mosquito inoculations were calculated by linear regression of the log_{10} PFU/mL to the viral RNA amount (Ct) obtained from the rRT-PCRs of the corresponding dilution series. Standard curves showed a high correlation coefficient for the CHIKV (Log_{10} PFU = −0.3129*Ct + 13.468; R^2 = 0.999) and DENV (Log_{10} PFU = −0.3149*Ct + 12.723; R^2 = 0.952).

Dissemination Efficiency and Viral Loads

Viral load measured in the pools of legs and wings from both irradiated and unirradiated mosquito species infected with

TABLE 1 | Number and percentage of positive pools of legs and wings (dissemination efficiency) and saliva (transmission efficiency) from irradiated (rad) and unirradiated (control) *Ae. albopictus* and *Ae. aegypti* females orally fed with CHIKV and DENV after 7- or 14-days incubation, respectively. The range of log_{10} PFU measured (MIN—MAX) for legs and wings and saliva pools in both irradiated and unirradiated species infected with CHIKV or DENV is also reported. Each pool of legs and wings (L&W) and saliva (SAL) homogenates were derived from eight females.

Virus	Status	Species	Pool (N)	Dissemination efficiency (L&W)			Transmission efficiency (SAL)		
				PCR/+ (N)	PCR/+ (%)	Log_{10} PFU (min—max)	PCR/+ (N)	PCR/+ (%)	Log_{10} PFU (min—max)
CHIKV	Control	*Ae. albopictus*	8	8	100%	5.99—6.58	8	100%	1.73—4.51
CHIKV	Rad	*Ae. albopictus*	12	12	100%	6.11—6.04	12	100%	1.66—4.24
CHIKV	Control	*Ae. aegypti*	10	10	100%	5.06—6.77	10	100%	1.16—3.59
CHIKV	Rad	*Ae. aegypti*	12	12	100%	5.50—6.25	12	100%	2.13—4.70
DENV	Control	*Ae. albopictus*	8	7	88%	3.21—4.33	2	25%	0.00—1.48
DENV	Rad	*Ae. albopictus*	11	7	64%	0.00—4.84	3	27%	0.00—2.45
DENV	Control	*Ae. aegypti*	9	6	67%	0.00—4.41	2	22%	0.00—2.29
DENV	Rad	*Ae. aegypti*	13	13	100%	2.88—4.25	1	8%	0.00—2.94

CHIKV or DENV is shown in **Table 1** (dissemination efficiency) and **Figures 1A,B**. All the pools of legs and wings from the mosquitoes infected with CHIKV were positive whereas for DENV, the percentage of positive pools ranged between 64 and 100% (**Table 1**). No differences were observed in the dissemination efficiency of irradiated and unirradiated mosquitoes infected with DENV at the pool level (χ^2 = 6.3695, df = 3, p = 0.095; **Table 1**). In the case of DENV, we did not observe differences between the mosquito species (**Figure 1A**, likelihood ratio = 3.167, p = 0.205) and radiation had no effect on the viral load measured in both species (likelihood ratio = 3.249, p = 0.197) (**Table 2**). In the case of CHIKV, however, the viral load measured in *Ae. albopictus* was higher (**Figure 1B**, likelihood ratio = 10.578, p = 0.001) and irradiation increased the virus load in both species (likelihood ratio = 41.470, $p < 10^{-4}$) (**Table 2**).

Transmission Efficiency and Viral Loads

Viral RNA presence was also confirmed among saliva collected from both irradiated and not-irradiated females on day 7 or day 14 after exposure to either CHIKV or DENV, respectively. Not all positive leg and wing pools were positive for viral RNA in the saliva. Nevertheless, 100% of saliva pools derived from *Ae. albopictus* and *Ae. aegypti* infected with CHIKV were positive, while only 8–27% of the saliva pools from females inoculated with DENV were positive with a lower PFU range (**Table 1**, transmission efficiency). Viral loads in pools of saliva from both irradiated and unirradiated mosquito species infected with CHIKV or DENV are shown in **Table 1** (transmission efficiency) and **Figures 1C,D**. No differences were observed in the transmission rate of irradiated and unirradiated mosquitoes infected with DENV (χ^2 = 1.2415, df = 3, p = 0.7431). For both viruses, we did not observe differences in the transmission rates between the mosquito species (likelihood ratio = 0.109, p = 0.742 for DENV and likelihood ratio = 0.273, p = 0.602 for CHIKV). Also, irradiation did not impact transmission rates (likelihood ratio = 0.172, p = 0.679 for DENV and Likelihood ratio = 3.493,315, p = 0.062 for CHIKV) (**Table 2**; **Figures 1C**,D).

A significant correlation was observed between the viral loads measured in the saliva pool samples and in the corresponding individual samples (t = 3.3424, df = 13; p = 0.0053; Cor estimated = 0.68). In DENV, the two mosquito species had a similar virus load (likelihood ratio = 4.343, p = 0.114) and irradiation only marginally increased transmission (likelihood ratio = 5.689, p = 0.058) (**Table 2**; **Figures 1E**,F). In CHIKV, no impact of mosquito species (likelihood ratio = 1.100, p = 0.294) nor irradiation (likelihood ratio = 1.522, p = 0.217) was observed (**Table 2**; **Figures 1E**,F).

DISCUSSION

This study demonstrated that the irradiation of female *Ae. aegypti* and *Ae. albopictus* as pupae with a dose of 40 Gy had no effect on the dissemination of DENV but increased the virus load of CHIKV in the mosquitoes' bodies in both species. However, irradiation did not increase the transmission rate of both viruses in both mosquito species, although a marginal increase was detected at the individual level for DENV in *Ae. albopictus*. Both species are well-known vectors of these two viruses, with dissemination and transmission rates ranging between 33% and 87% for DENV (Vazeille et al., 2010; Guo et al., 2013; Gonçalves et al., 2014) and between 40% and 100% for CHIKV (Schuffenecker et al., 2006; Richards et al., 2010; Vega-Rúa et al., 2014), respectively. The highest virus dissemination of CHIKV was recorded in *Ae. albopictus*, regardless of irradiation treatment. The CHIKV strain used here is the natural mutant form with one amino-acid substitution (alanine → valine) in position 266 of the E1 glycoprotein (E1-A226V) (Schuffenecker et al., 2006). It was previously demonstrated that this strain is better adapted to *Ae. albopictus* than to *Ae. aegypti*, resulting in a high replication level and a shorter extrinsic incubation period (EIP) for both species, with infectious virus particles being detectable in the saliva as early as two dpi (Dubrulle et al., 2009). However, despite the larger dissemination efficiency observed in *Ae. albopictus* compared with *Ae. aegypti*, we did not observe a clear increase of CHIKV viral load between the saliva of the two species in either pools or individuals confirming the essential role of salivary glands in selecting viruses for efficient

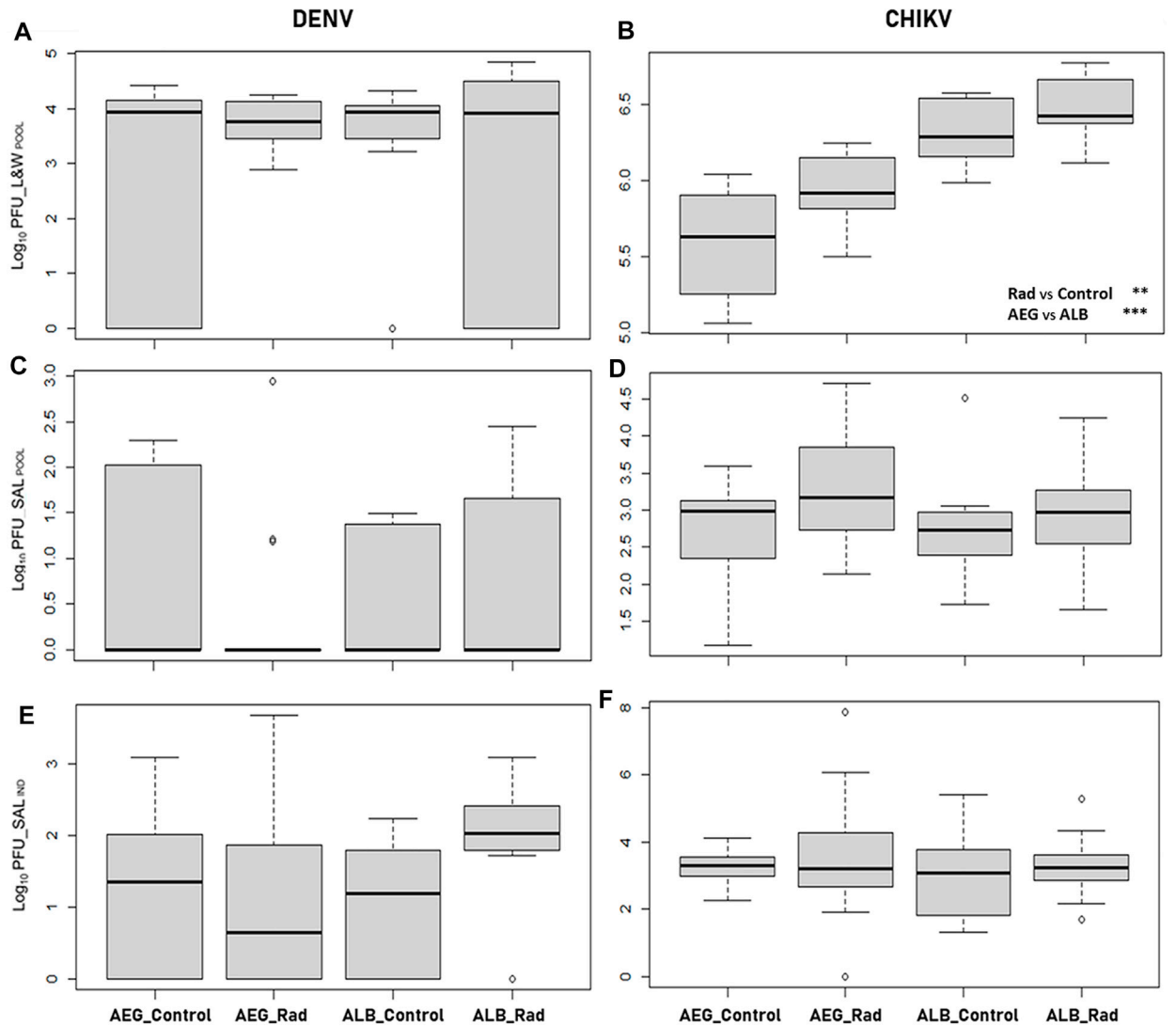

FIGURE 1 | Boxplots of the viral load (log_{10} PFU) measured in pooled samples (POOL) of legs and wings (L&W) and saliva (SAL) from both irradiated (rad) and unirradiated (control) *Ae. aegypti* (AEG) and *Ae. albopictus* (ALB) mosquitoes infected with dengue (DENV, 14 dpi; or **(A,C)** chikungunya virus (CHIKV, 7 dpi; **(B,D)** Each pool sample consisted of homogenates of legs and wings and saliva from eight individual females. Viral load measured in saliva samples from individual females (IND) is also reported from both irradiated and not-irradiated *Ae. aegypti* and *Ae. albopictus* mosquitoes infected with (E) dengue or **(F)** chikungunya virus. There are no statistical differences in the different groups represented in the figure except for the significant effects of radiation (rad) and the species used (AEG or ALB) on the dissemination of chikungunya virus as shown in **(B)** and reported in **Table 2**. Asterisks represent statistical significance (**$p < 0.01$; ***$p < 0.001$).

TABLE 2 | Fixed effects of linear mixed-effects models fit by maximum likelihood for the effect of the virus type (CHIKV and DENV) and radiation treatment (rad) on female viral loads measured in legs and wings and in the saliva on pooled (pool) and individual samples (individual). Values have been presented considering the two mosquito species together except for data on CHIKV presence in legs and wings (*) where the mosquito species and the radiation treatment were both kept in the best model.

Virus		Origin	Sample	Factors	Value	SE	df	t-value	*p* value
CHIKV	*	Legs and wings	Pool	Rad	0.261	0.078	28	3,342	0.002
	*	Legs and wings	Pool	*Ae. albopictus*	0.628	0.078	28	8,105	0.000
	—	Saliva	Pool	Rad	0.423	0.231	29	1,831	0.077
	—	Saliva	Individual	Rad	0.366	0.298	46	1,227	0.226
DENV	—	Legs and wings	Pool	Rad	0.296	0.523	27	0.566	0.576
	—	Saliva	Pool	Rad	–0.121	0.296	27	–0.408	0.687
	—	Saliva	Individual	Rad	0.420	0.283	41	1,483	0.146

transmission (Vega-Rúa et al., 2014). Ct values of DENV-inoculated mosquitoes were generally higher than those of CHIKV-inoculated mosquitoes. Still, the initial viral dose of CHIKV (8.0 log10TCID50/mL) in the inoculum was 2 logs higher than the one used for DENV (6.0 log10TCID50/mL). However, DENV and CHIKV particles were well disseminated through the entire body with clear virus amplification between the initial intake of the infectious inoculum (day 0 females) and the end of the EIP (7 or 14 dpi for CHIKV and DENV, respectively) in both irradiated and unirradiated mosquitoes. Despite the high inoculum of CHIKV used for the females' infections, it was still possible to observe a different pattern in the amplification of the virus at the dissemination stage (legs and wings), with a higher virus load among irradiated females of both species while irradiation treatment had no significant effect on viral load amplification in the salivary glands.

Exposure to ionizing radiation is the most used method to sterilize male insects in AW-IPM programs that include an SIT component (Bakri et al., 2005). Because irradiation may adversely influence the quality of the insect to be released through mutations in the somatic cells, it is essential to select the optimum radiation dose that effectively sterilizes the insect without adversely affecting the insect's competitiveness (Bakri et al., 2005; Parker et al., 2021). Dose-dependent radiation damage to the insect's mid-gut has been previously identified as the main factor affecting changes in the infection levels and in the alteration in the gut bacterial communities (Lauzon and Potter, 2012; Woruba et al., 2019; Guissou et al., 2020). The structural damages observed in the mid-gut tissues of irradiated flies lead to metabolic and physiological abnormalities (Lauzon and Potter, 2012) and could be related to the increased dissemination efficiency observed in irradiated female mosquitoes in this study. Microscopic observations on fruit flies irradiated at the dose used in the SIT suggested that radiation interfered with tissue formation and generated small breaks in the peritrophic membrane integrity, possibly affecting the gut functions, the passage of pathogens, and the overall quality and competitiveness in nature (Lauzon and Potter, 2012). Recently, it was reported that micro-perforations produced after consecutive blood feedings in the virus-impenetrable mid-gut basal lamina provided a mechanism for enhanced virus escape increasing dissemination and transmission efficiency for DENV and CHIKV in *Ae. albopictus* and *Ae. aegypti* (Armstrong et al., 2020). Although the impact of irradiation on mid-gut virus infection was not the topic of this work, our data indicate that an irradiation dose of 40 Gy delivered at the pupal stage increased the capability of the CHIKV variant E1-226V to overcome the intrinsic barriers of orally infected mosquitoes while we did not observe any increase in virus transmission in the irradiated mosquito for both species. The key role of the mid-gut barrier in CHIKV E1-226V dissemination through the mosquito body has been already demonstrated in *Ae. albopictus* in comparison with *Ae. aegypti* (Arias-Goeta et al., 2013) and possible damage in the mid-gut barriers following irradiation could therefore support the different CHIKV E1-226V dissemination efficiency observed in our trial between the two species.

The mid-gut constitutes an important component of the mosquito's immune response defense against transmitted pathogens and it is the first barrier that viruses must cross to achieve a successful viral cycle (Janeh et al., 2017; Taracena et al., 2018). The effectiveness of an early mid-gut cellular renewal and the maintenance of mid-gut homeostasis during viral infection proved to be an important factor in the antiviral response of *Ae. aegypti* against DENV infections (Dennison et al., 2014; Taracena et al., 2018; Janeh et al., 2019). Because radiation-induced cellular damage could affect the viral permeability of the mid-gut membranes and delays the activation of the regenerative cellular process (Lauzon and Potter, 2012; Taracena et al., 2018), there may be a larger window of opportunity during which the mid-gut membrane of the irradiated mosquito becomes permissive for viral dissemination (Dong et al., 2017). The peculiar viral replication kinetics in the CHIKV strain (Merwaiss et al., 2021), with a shorter EIP, could have played a crucial role in increasing mid-gut escape and dissemination efficiency in irradiated mosquitoes in comparison with DENV in our trials. The marginal, not significant, increase of the viral load observed in *Ae. albopictus* females infected with DENV would be worth investigating in further studies.

In addition to the maintenance of an effective vector competence, the irradiated females accidentally released during an SIT campaign must effectively disperse, feed, and survive in the field to increase the risk of transmission of arboviral diseases. The mean survival rates observed during mark-release-recapture studies with irradiated and unirradiated *Aedes* males were similar with a mean lifespan estimated at two to eleven days (Muir and Kay, 1998; Bellini et al., 2010; Brady et al., 2013; Vavassori et al., 2019). As the more radiosensitive female mosquitoes have shorter survival than their male counterparts under laboratory conditions (Bond et al., 2019; Aldridge et al., 2020), further investigations will be required to estimate if irradiated females are capable of surviving the EIP to become infectious under field conditions. *Aedes albopictus* and *Ae. aegypti* female mosquitoes would need to survive six to twelve days in the field to acquire and effectively transmit arboviruses (Tjaden et al., 2013; Christofferson et al., 2014). Therefore, the average lifespan of irradiated *Aedes* females may not be sufficient to consider them effective vectors. Moreover, the reduced survival and blood-feeding frequency observed in irradiated *Aedes* females (Bond et al., 2019; Cunningham et al., 2020), and the increased mortality resulting from the ingestion of an infected blood meal (Maciel-de-Freitas et al., 2011; da Silveira et al., 2018), can have a significant impact on the overall vectorial capacity and transmission efficiency of irradiated *Aedes* females accidentally released in an operational *Aedes* SIT program. In the present study, only one time point of infection was investigated (days 7 and 14 for CHIKV and DENV, respectively) with relatively small sample sizes. Further studies would be therefore required to explore the temporal dynamics of viral dissemination and transmission in irradiated female mosquitoes fed with virus-spiked blood, that is, at daily intervals and with different radiation doses. This will reveal whether irradiation has an impact on the EIP as this could significantly influence the epidemiology of vector-borne diseases in the field. Moreover, the correlation between viral infection, mid-gut cell damages, and the alteration of the gut microbiota in relation to

different radiation doses need to be further addressed to investigate the impact of radiation on female vector competence and male survival, flight ability, and field competitiveness.

This study showed that irradiation did not significantly alter the competence of irradiated female *Aedes* mosquitoes, illustrating the need for improved, more fail-proof sexing systems to reduce female contamination in released sterile males to enhance the acceptability of SIT programs and prevent any transmission risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All experiments were performed in accordance with relevant named guidelines and regulations.

AUTHOR CONTRIBUTIONS

FB, MV, and EV designed the experiments and conceived the project. FB and EV carried out virus-mosquito experiments, dissections, and molecular studies. FB, JB, and EV processed the experimental data. EV supervised the work. All authors drafted, reviewed, and approved the final manuscript.

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Gene Editing and Genetic Control of Hemipteran Pests: Progress, Challenges and Perspectives

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The origin of the order Hemiptera can be traced to the late Permian Period more than 230 MYA, well before the origin of flowering plants 100 MY later in during the Cretaceous period. Hemipteran species consume their liquid diets using a sucking proboscis; for phytophagous hemipterans their mouthparts (stylets) are elegant structures that enable voracious feeding from plant xylem or phloem. This adaptation has resulted in some hemipteran species becoming globally significant pests of agriculture resulting in significant annual crop losses. Due to the reliance on chemical insecticides for the control of insect pests in agricultural settings, many hemipteran pests have evolved resistance to insecticides resulting in an urgent need to develop new, species-specific and environmentally friendly methods of pest control. The rapid advances in CRISPR/Cas9 technologies in model insects such as *Drosophila melanogaster*, *Tribolium castaneum*, *Bombyx mori,* and *Aedes aegypti* has spurred a new round of innovative genetic control strategies in the Diptera and Lepidoptera and an increased interest in assessing genetic control technologies for the Hemiptera. Genetic control approaches in the Hemiptera have, to date, been largely overlooked due to the problems of introducing genetic material into the germline of these insects. The high frequency of CRISPR-mediated mutagenesis in model insect species suggest that, if the delivery problem for Hemiptera could be solved, then gene editing in the Hemiptera might be quickly achieved. Significant advances in CRISPR/Cas9 editing have been realized in nine species of Hemiptera over the past 4 years. Here we review progress in the Hemiptera and discuss the challenges and opportunities for extending contemporary genetic control strategies into species in this agriculturally important insect order**r.**

Keywords: Hemiptera, CRISPR/Cas9, whiteflies, gene editing, mutations, genetic control

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INTRODUCTION

Annually, insect pests decimate agriculture. The direct damage caused by pest feeding decreases the quality and yields of food, fiber, feed, and forage crops. Furthermore, the ability of some pests to vector phytopathogenic viruses and microbes further compromises agricultural productivity. Plant diseases and invasive pests cause an estimated $290 billion of loss to the global economy with losses caused by pests ranging from 20 to 40% of annual global crop production (FAO, 2019). While deployment of integrated pest management strategies limit losses, the current reliance on chemical

insecticide applications and the emergence of insecticide-resistant pests has emphasized the pressing need for development of sustainable and environmentally sound practices for insect control, such as genetic control. Genetic-control strategies are species specific and designed to eradicate or replace insect pest populations, thereby providing an additional set of tools for effective integrative pest management. The principal targets of contemporary approaches for genetic control of pest insects have been dipteran and lepidopteran species. While these genetic-control strategies are advanced and, in some cases, almost ready for field deployment, significant hurdles remain (Hammond et al., 2021).

Most surprisingly, genetic control strategies for the Hemiptera are currently lacking, despite the importance of many hemipteran species as agricultural pests. Of particular importance to global agriculture are the sap-feeding species within the order Hemiptera. For example, the whitefly *Bemisia tabaci* is one of the top 100 insect pests world-wide with a broad host plant range (https://stateoftheworldsplants.org/2017/report/SOTWP_2017.pdf, Lowe et al., 2000. http://www.iucngisd.org/gisd/100_worst.php). The importance of phytophagous hemipteran pests is emphasized by trends in the literature. Of the 1,187 arthropod pests publications between 2012–2016 (Willis, 2017), four hemipteran species ranked in the top ten with: *B. tabaci* (#2), green peach aphid (*Myzus persicae*, #7), cotton aphid, (*Aphis gossypii*, #9), and brown planthopper (*Nilaparvata lugens*, #10). In addition, there was a significant increase in the number of publications focusing on insect pests of plants from 2007–2011 to 2012–2016; again, five of the top ten species were hemipteran. Our survey of publications identified in the National Library of Medicine at NIH and Web of Science identified over 128,367 and 135,944 publications on the Hemiptera, respectively, from 2012–2022 (**Figure 1**). In addition, many hemipteran species are invasive pests of agriculture. Members of the Hemiptera are the most abundant of non-native insect species in North America with near to 800 species of Hemiptera introduced since 1800 (Yamanaka et al., 2015; MacLachlan et al., 2021).

For both model and non-model insects, the enabling genetic tools derived from CRISPR/Cas9 gene-editing technologies have revolutionized insect biotechnology. CRISPR/Cas9 technologies enable efficient, cost-effective, precision mutagenesis that has been leveraged for improved and elegant strategies proposed for the genetic control of insect pests (Kyrou et al., 2018; Kandul et al., 2019; Hammond et al., 2021; Kandul et al., 2021; Li et al., 2021; Meccariello et al., 2021). Genetic-control strategies for all insect pests are completely dependent on the ability to genetically modify, through transgenesis or paratransgenesis, the target insect species and mass rearing of the target insect for deployment of these technologies in the field. Both have presented challenges in the Hemiptera. Genetic-control strategies for the mosquito *Anopheles gambiae* are being developed and cage tested (Hammond et al., 2021). Field release of genetically modified strains of *Aedes aegypti* that achieve population reduction has occurred in the Caribbean, Brazil, and the Florida Keys (Erickson, 2016; Schairer et al., 2021). Finally, a genetically modified strain of *Plutella xylostella* has been released in central New York to eliminate the local populations of this pest (Waltz, 2015).

The first genetic-control strategy to be deployed was the Sterile Insect Technique (SIT). SIT depends on the large-scale release of sterile males of a target species. Matings with females in the field are infertile, ultimately leading to target population decline (Bushland et al., 1955). SIT can control livestock and crop losses by eliminating these pest insects. For example, deposition of eggs within fruit by female fruit flies leads to the development and subsequent feeding of larvae causing massive damage and post-harvest yield loss; SIT reduces viable eggs, limits fruit damage and affords pest control. Other proposed genetic-

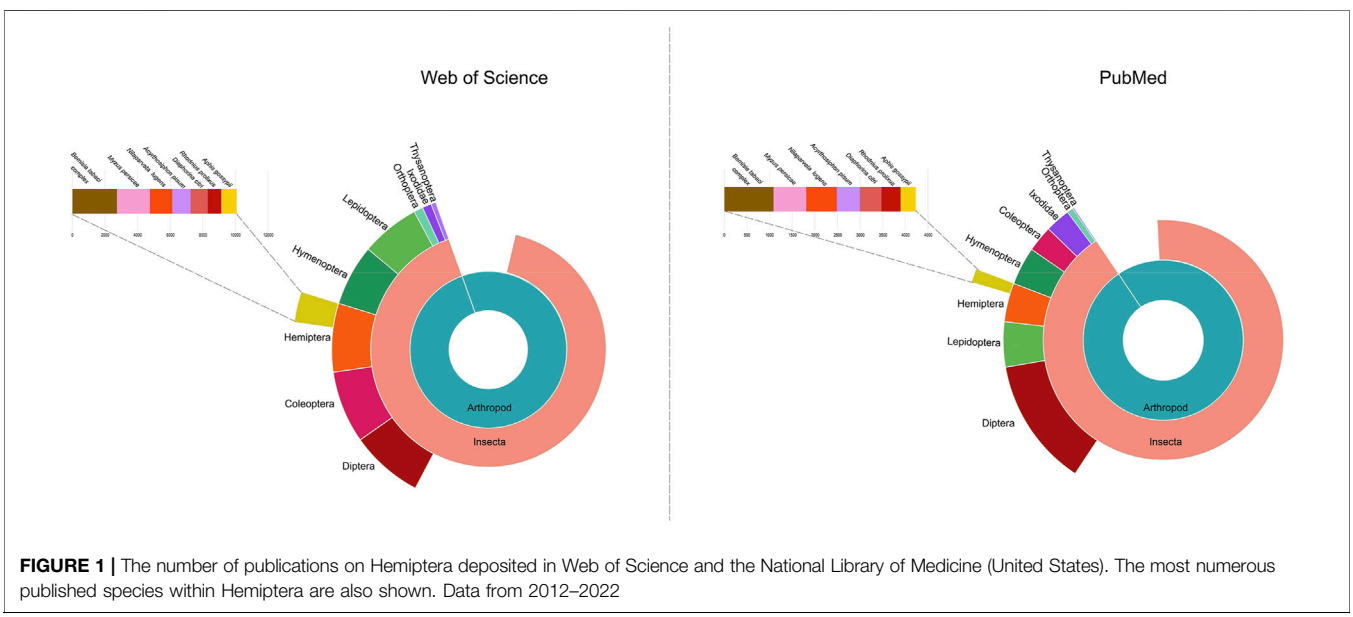

FIGURE 1 | The number of publications on Hemiptera deposited in Web of Science and the National Library of Medicine (United States). The most numerous published species within Hemiptera are also shown. Data from 2012–2022

control strategies primarily target the viability of females in the target species (Kyrou et al., 2018). As the number of females decline, there is a subsequent reduction in the overall population and prevention of pathogen vectoring. Pathogen vectoring is often associated with the activities of females; for example, female mosquitoes that transmit human pathogens when taking blood meals. SIT and other genetic-control strategies have been developed for insects that share a core of features that make them uniquely adapted to these genetic control mechanisms. These features include: exclusive sexual reproduction, males not being directly responsible for economic or medical impacts, cost-efficient mass-rearing, the ability to use antibiotics to regulate genetic control systems, and well-developed methods and tools for engineering insect genomes (Lance and McInnis, 2005; Robinson, 2005; Leftwich et al., 2014).

Unfortunately, target insect features that are essential for current SIT and genetic-control strategies in the Diptera and Lepidoptera are not always present in species of the Hemiptera. First and foremost, for the Hemiptera, both males and females can vector disease-causing pathogens and both cause significant feeding damage to plant hosts (Hogenhout et al., 2008). For example, *B. tabaci* males and females can acquire and transmit Tomato Yellow Leaf Curl Virus (Ning et al., 2015). For this reason, control methods that solely target males (i.e., irradiation for SIT) or females may not be applicable. Furthermore, little is known about the molecular basis of sex determination in Hemiptera. In the mosquito *An. gambiae*, the Mediterranean fruit fly *Ceratits capitata*, and the diamondback moth, *P. xylostella*, the mechanisms of sex determination have been determined at sufficient depth to enable the sex ratio to be modified (Fu et al., 2007; Jin et al., 2013; Kyrou et al., 2018; Meccariello et al., 2021). This is a critical step for control strategies dependent on the elimination of females. Unfortunately, this level of understanding is currently lacking for the Hemiptera with the exceptions of the brown planthopper (*N. lugens)*, the whitefly (*B. tabaci)*, and the kissing bug (*Rhodnius prolixus).* For these species, some of the key genes in sex determination have been identified and the differential splicing of their transcripts between the sexes determined but more research is needed before sex ratios can be altered as part of a genetic-control strategy (Xie et al., 2014; Guo et al., 2018; Zhuo et al., 2018; Wexler et al., 2019; Zhuo et al., 2021). In addition, some hemipteran species, such as whiteflies, are haplo-diploid and others lack Y chromosomes (Pal and Vicoso, 2015; Blackmon et al., 2017). These alternative genetic systems will, most likely, influence the design and efficiencies of genetic-control mechanisms (Champer et al., 2020; Li J. et al., 2020).

Second, cost-efficient mass rearing is not commonplace in the Hemiptera. Large-scale rearing has a large physical footprint making such initiatives space-, time- and cost-intensive. Third, phytophagous Hemiptera harbor obligate endosymbiotic bacteria, making the use of antibiotics to induce or repress transgene expression problematic. This negates the use of the popular tetracycline on/off bacterial system that has been used to regulate transgene expression in other insects (Thomas et al., 2000; Gong et al., 2005; Jin et al., 2013). Fourth, some Hemiptera have complex and environmentally regulated lifecycle features that make them challenging species for any technology that is dependent on sexual crosses. For example, many aphids reproduce parthenogenetically and the synchronous production of males and oviparous females is triggered by environmental cues (often only once per year) (Simon and Peccoud, 2018); this can be difficult to achieve in the laboratory, representing a significant technical challenge. In addition, other hemiptera have long generation times and significant annual diapause periods (Simon and Peccoud, 2018; Krugner et al., 2019). Fifth, the genetic toolbox required for routine insect biotechnology and the methods for introducing macromolecules (i.e., DNAs, RNAs, and proteins) are currently underdeveloped. Few constitutive and tissue-, cell- or stage-specific promoters and other regulatory elements have been isolated and shown to be active in Hemiptera. Finally, the methods for CRISPR/Cas gene editing are just emerging and standard methods for gene introduction has yet to be fully explored.

While many of the attributes that make Diptera and Lepidoptera amenable to SIT and other control strategies are not present in the Hemiptera, many of these constraints are not insurmountable. Current control strategies will need to be adapted or new strategies developed to enable the field of hemipteran genetic control. Within the past 4 years, there have been substantial advances in the field of hemipteran biotechnology. The enabling technology of CRISPR/Cas-mediated mutagenesis in the Hemiptera is emerging. The increasing numbers of annotated genome assemblies now provide essential components for the development of the genetic toolbox required for extending genetic control into hemipteran pests. Here we review the recent progress that has been achieved in extending CRISPR/Cas9 gene-editing technology into hemipteran species and offer perspectives on how these technologies may be further developed into genetic-control strategies for use in the field.

Genome Assemblies of Hemiptera

Accurate genomic information is critical for the development of genome-editing strategies. Well-annotated genomes facilitate the identification of target genes, the design of gene-specific sgRNAs (single-guide RNAs) and the identification of off-target sequences that could compromise the specificity of mutagenesis. Of the 26 insect orders, eight have genome projects. At present, there are 2,790 insect genomes accessible at NCBI (ncbi.nlm.nih.gov) and 114 are from the Hemiptera, representing 63 different species (**Figure 2**). The Hemiptera rank fifth, trailing the Lepidoptera, Diptera, Hymenoptera, and Coleoptera. When the genomes of insect pests of US agriculture are considered (i5k, 2022), the Coleoptera and Hemiptera predominate (**Figure 2**). Currently, a minute amount of the genomic diversity of the Hemiptera has been captured as more than 86,000 hemipteran species have been identified (ELO, 2022). The number of completed hemipteran genome projects has risen markedly since 2018, with chromosome-level genome assemblies first appearing in 2018 and increasing each year thereafter (**Figure 3**; **Table 1**). For the *B. tabaci* species complex, there are ten genomes available and, for nine other Hemiptera, there are three or more independent

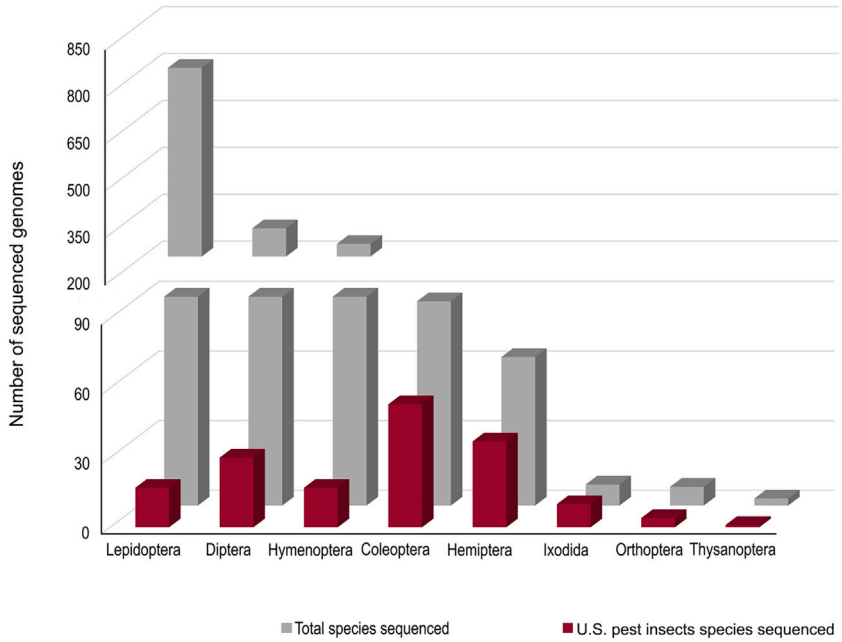

FIGURE 2 | The number of genome projects for each species within each insect order based on depositions at the NCBI (all species) and at the i5K project (http://i5k.github.io/about). Data as at 2022.

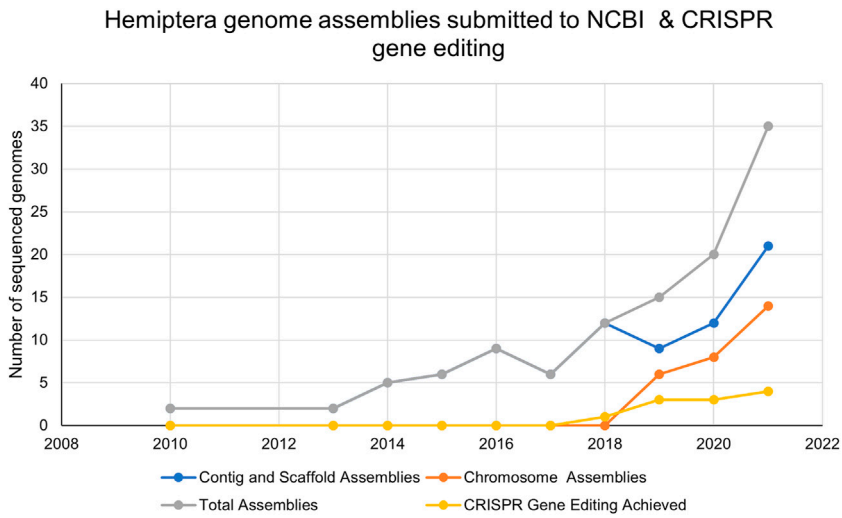

FIGURE 3 | The number of Hemiptera genome assemblies at the NCBI and the number of publications reporting CRISPR/Cas9 mutagenesis in Hemiptera from 2010–2021.

genomes (**Figure 4**, **Supplementary Table S1**). This provides insights into the sequence diversity associated with hemipteran species complexes and biotypes. All but one of these species (the bed bug, *Cimex lectularis*) are significant agricultural pests. *B. tabaci*, *N. lugens*, *Acyrthosiphon pisum* (pea aphid), and *Homalodisca vitripennis* (glassy-winged sharpshooter) are discussed in more detail in this review, as their genomes have been leveraged for CRISPR/Cas9-mediated gene editing.

Of the 114 available hemipteran genomes (contigs, scaffolds, or chromosome-level assemblies) (**Supplementary Table S1**), only 25 contig/scaffold assemblies and 16 chromosome-level genomes are published (**Table 1**; **Figure 3**). A hemipteran genome explosion is beginning as 10 additional chromosome-level assemblies should be published soon (**Supplementary Table S1**). The high-confidence chromosome genome assemblies together with determination of chromosome number, the sex

TABLE 1 | Chromosomal assembly genome projects in Hemiptera deposited at NCBI.

Suborder	Family	Organism name	Number of Genes	Chromosome Number (including Sex Chromosome)	Sequenced Sex Chromosome (Sex Determination)	Mitochondrial Chromosome	References
Auchenorrhyncha	Delphacidae	*Laodelphax striatellus* (small brown planthopper)	16,412	15	X (X0)	YES	Noda and Tatewaki, (1990), Song and Liang, (2009), Ma et al. (2021)
		Nilaparvata lugens (Brown planthopper)	21,385	16	X,Y (XY)	YES	Song and Liang, (2009)
Heteroptera	Alydidae	*Riptortus pedestris* (bean bug)	21,562	6	X (X0)	YES	Hua et al. (2008), Kaur et al. (2012), Huang et al. (2021)
	Miridae	*Apolygus lucorum* (Mirid Bug)	20,353	17	-	YES	Liu et al. (2021)
		Cyrtorhinus lividipennis	14,644	13	X,Y (XY)	YES	Bai et al. (2022)
	Pentatomidae	*Aelia acuminata* (Bishop's Mitre)	-	8	X,Y (XY)	YES	Crowley and Barclay (2021)
Sternorrhyncha	Aphalaridae	*Pachypsylla venusta* (hackberry petiole gall psyllid)	19,976	12	X (X0)	-	Yiyuan Li et al. (2020)
	Aphididae	*Acyrthosiphon pisum* (Pea aphid)	21,915	4	X (X0)	YES	Mandrioli et al. (2011), Li et al. (2019)
		Aphis gossypii (cotton aphid)	15,188–18,245	4	X (X0)	YES	Jaquiery et al. (2012), Zhang et al. (2022)
		Eriosoma lanigerum (woolly apple aphid)	28,186	6	X (X0)	YES	Biello et al. (2021)
		Hormaphis cornu (Witch-hazel cone gall aphid)	19,582	9	- (X0)	-	Korgaonkar et al. (2021)
		Metopolophium dirhodum (rose-grain aphid)	18,003	9	X (X0)	-	Zhu et al. (2022)
		Rhopalosiphum maidis (green corn aphid)	17,629	4	-(X0)	YES	Chen et al. (2019)
		Sitobion miscanthi (Indian grain aphid)	16,006	15	X (X0)	YES	Jiang et al. (2019)
	Pseudococcidae	*Phenacoccus solenopsis* (cotton mealybug)	11,880	5	-	-	Meizhen Li et al. (2020)

chromosomes, and gene numbers provide a foundation for the identification of gene targets and sgRNA design necessary for CRISPR/Cas gene editing (**Table 1**). As the number of completed and well-annotated hemipteran genome projects increases and genomes are re-sequenced to capture diversity within a species or species complex, the opportunities to conduct genetic research in these species will increase. It will be critical that these genome projects be of high quality in terms of the depth and breadth of coverage so that sgRNAs specific to unique target sites can be designed with confidence. Even with the existence of a reference genome for a given species, a laboratory, or local population of a species may need to be sequenced, at least across proposed target sites, in order to ensure that single-nucleotide polymorphisms (SNPs) do not confound sgRNA efficiency.

Gene Delivery in Hemiptera

Two technologies have been used to assess hemipteran gene function: RNA interference (RNAi) and CRISPR/Cas9 gene editing. For RNAi, double-stranded RNAs (dsRNAs) are delivered to insects to transiently knock-down target gene expression yielding partial to full loss-of-function mutants. Delivery of dsRNAs to Hemiptera has been achieved by

TABLE 2 | The gene targets and efficiencies of CRISPR/Cas9-mediated mutagenesis in Hemiptera.

Species (common name)	Genome Available at NCBI?	Gene Target	Frequency of KO	Initial Technique of Detection	Frequency of G1 Gene Editing	Mutant G2 Generation Produced?	References
Nilaparvata lugens (Brown planthopper)	YES	*cinnabar*	0	Visual	48.8%	Not described	Xue et al. (2018)
		white	27.3%	Visual	3.2%	Not described	Xue et al. (2018)
		Insulin receptor 1	~1–11.2% (plasmids) Not reported (Cas9 protein + sgRNA)	Deep sequencing of amplicons	9.1–35.7% (plasmids) 50–100% (Cas9 protein + sgRNA)	Not described	Zhao et al. (2019)
		Insulin receptor 2	Not described	Not described	Not described	Not described	Xue et al. (2021)
		NICSAD	9.5%	Sequencing	36.3% heterozygous	13.1% homozygous/ 53.3% heterozygous	Chen et al. (2021)
Peregrinus maidis (Corn planthopper)	NO	*white*	0.324	Visual	0	Not described	Klobasa et al. (2021)
Homalodisca vitripennis (Glassy-winged sharpshooter)	YES	*cinnabar*	58.9–66.7%	Visual	100%	100%	Pacheco et al. (2022)
		white	61.2–80.0%	Visual	100%	100%	
Euschistus heros (Neotropical stink bug)	YES	*yellow*	33.3%	Visual	0	Not described	Cagliari et al. (2020)
Lygus hesperus (Western tarnished plant bug)	NO	*cardinal*	100%	Visual	91.2%	A mix of wild-type and mutants	Heu et al. (2022)
		cinnabar	40–100%	Visual	77.9%	A mix of wild-type and mutants	
Oncopeltus fasciatus (Milkweed bug)	YES	*white*	14.0–92.5%	Visual	64.6% heterozygotes	No white homozygotes	Reding and Pick, (2020)
Pyrrhocoris apterus (Linden bug)	NO	*Cryptochrome 2*	35.7%	Heteroduplex assays	1.4–7.4% (1 sgRNA)	Not described	Kotwica-Rolinska et al. (2019)
		timeless	35.4%		4.2–66.7% (2)		
		Period	42.8%		0–61.5% (4)		
		pigment dispersing factor	83.3%		10.0–77.3% (2)		
		TEFLamide	43.3%		2.7–35.7% (2)		
Acyrthosiphon pisum (Pea aphid)	YES	*stylin-01*	66.7%,77.8%	PCR	35.3%	Not described	Le Trionnaire et al. (2019)
Bemisia tabaci (Whitefly)	YES	*white*	0.2–2.5%	Visual	Heterozygotes	21.4%	Heu et al. (2020)

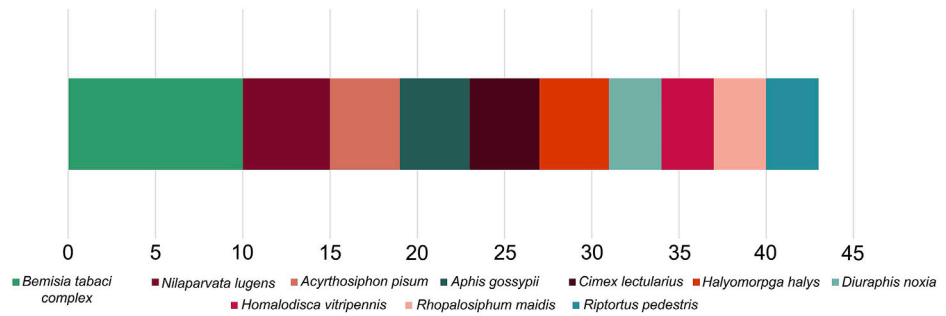

FIGURE 4 | The ten most sequenced species (or species complex) within the Hemiptera with the number of submissions for each shown, based on NCBI data.

TABLE 3 | Microinjection protocols of CRISPR/Cas9-mediated mutagenesis in Hemiptera.

Species (common name)	Gene Target	Needle Type	Cas9 Protein (P) or mRNA (M) Concentration (ng/μL)	sgRNA or crRNA/ trRNA Concentration (ng/μL)	Gene, Plasmid Concentration (ng/μL)	Dye	References
Nilaparvata lugens (Brown planthopper)	*cinnabar*	Glass	500 (M)	150 (1 sgRNA)	-	-	Xue et al. (2018)
	white	Glass	500 (M)	400 (1 sgRNA)	-	-	Xue et al. (2018)
	Insulin receptor 1	Not described	200 (P)	50 each separately (3 sgRNAs)	vasa-Cas9 (300)	0.2% phenol red	Zhao et al. (2019)
	Insulin receptor 2	Glass	500 (M)	Not described	-	-	Xue et al. (2021)
	NlCSAD	Not described	200 (P)	50 (1 sgRNA)	U6a or U6b sgRNA (100)	0.2% phenol red	Chen et al. (2021)
Peregrinus maidis (Corn planthopper)	*white*	Quartz, beveled	500 (P)	400 each combined (3 sgRNAs)	-	20% phenol red	Klobasa et al. (2021)
Homalodisca vitripennis (Glassy-winged sharpshooter)	*White cinnabar*	Quartz, beveled	300 (P)	300 (1 sgRNA)	-	-	Pacheco et al. (2022), Al-Wahaibi and Morse (2009)
Euschistus heros (Neotropical stink bug)	*yellow*	Glass	300 (P)	300 (1 sgRNA)	-	-	Cagliari et al. (2020)
Lygus hesperus (Western tarnished plant bug)	*Cardinal cinnabar*	Quartz, beveled	300 (P)	150 each combined (2 sgRNAs)	-	-	Heu et al. (2022)
Oncopeltus fasciatus (Milkweed bug)	*white*	Borosilicate, tip opened with fine dissection scissors	300 (P)	80 each separately (3 sgRNAs)	-	-	Reding and Pick, (2020)
Pyrrhocoris apterus (Linden bug)	*Crypto-chrome 2* *timeless* *Period* *pigment dispersing factor* *TEFLamide*	Borosilicate, tip opened by gentle scratching with fine forceps	400 (M), 500 (P) 500 (P) 400 (M), 250, 500 (P) 250, 400, 500 (P) 400 (M), 250, 500 (P)	200, 400 (1 sgRNA) 200 (1 sgRNA) 200, 500 (1 sgRNA) 200 or 400 (1crRNA)/ 119 or 238 (1 trRNA), 200, 500 (1 sgRNA) 200 or 400 (1 crRNA)/ 119 or 238 (1 trRNA 200, 500 (1 sgRNA)	-	-	Socha, (1993), Kotwica-Rolinska et al. (2019)
Acyrthosiphon pisum (Pea aphid)	*stylin-01 (cuticular)*	Eppendorf Femtotips	333 (P)	40 each (4 sgRNAs)	-	-	Lin et al. (2014), Le Trionnaire et al. (2019)
Bemisia tabaci (Whitefly)	*white*	Quartz	BtKV-Cas9	250 of each combined (5 sgRNAs)	-	-	Heu et al. (2020)

numerous techniques including: microinjection, artificial diets, petiole dips, and topical application. The successes and challenges associated with dsRNA strategies have been recently reviewed so will not be discussed here (Jain et al., 2020; Jain et al., 2021). In contrast, CRISPR/Cas-editing technologies can generate gene-specific mutations that are heritable and are often loss-of-function mutations (Jinek et al., 2013; Gratz et al., 2013; Yu et al., 2013; Bassett et al., 2013; Kistler et al., 2015; Meccariello et al., 2017; Wei et al., 2014; Jinek et al., 2012). Methods for efficient macromolecule delivery are essential to any CRISPR genome-editing system and the delivery strategy can be a substantial technological hurdle preventing deployment in target organisms. While temporally delayed by 3 to 5 years relative to the gene-editing advances in *D. melanogaster*, mosquitoes, the Lepidoptera, and the Coleoptera, CRISPR/Cas9-editing is now reported for nine species of Hemiptera with a total of 17 different genes being targeted (**Tables 2–4**) (Xue et al., 2018;

TABLE 4 | Post-injection protocols of CRISPR/Cas9-mediated mutagenesis in Hemiptera.

Species (common name)	Gene Target	Time to Cellular Blastoderm	Time of Embryo Microinjection (h after oviposition)	Support Platform	Post-injection Treatment	Days to Hatch	References
Nilaparvata lugens (Brown planthopper)	*Cinnabar white*	>4 h at 26°C	1–2 h	Glass slide to which aligned embryos were affixed by dissolved glue from double-sided sticky tape	Placed in Petri dishes and covered with moist filter paper rinsed in 20 ng/μL tebucanazole and 50 ng/μL kanamycin and placed in a walk-in chamber	9	Xue et al. (2018)
	Insulin receptor 1	>4 h at 26°C	2–3 h	Double-sided sticky tape, desiccated for 2–4 min, then covered with halocarbon 700 oil	Placed in a plastic slide box containing a moist paper towel. At 6–7 days developed embryos were transferred to Kimwipes and the mineral oil removed. Embryos were placed in rice sheaths	6–11	Zhao et al. (2019)
	Insulin receptor 2	>4 h at 26°C	1–2 h	Glass slide to which aligned embryos were affixed by dissolved glue from double-sided sticky tape	Placed in Petri dishes and covered with moist filter paper rinsed in 20 ng/μL tebucanazole and 50 ng/μL kanamycin and placed in a walk-in chamber	9	Xue et al. (2021)
	NICSAD	>4 h at 26°C	<2 h	Double-sided sticky tape, desiccated for 2–4 min, then covered with halocarbon 700 oil	Placed in a humidity chamber in a plant growth incubator	6–7	Chen et al. (2021)
Peregrinus maidis (Corn planthopper)	*white*	Not described	16 h	Double-sided sticky tape on a coverslip placed on 1% agar	Transfer coverslip with embryos to fresh 1% agar plate and place in humidity chamber	8	Klobasa et al. (2021)
Homalodisca vitripennis (Glassy-winged sharpshooter)	*Cinnabar white*	Undifferentiated stage lasts for ~90 h	2 h	Sorghum leaf disc containing egg mass placed on 1% phytoagar	Leaf disc on phytogar placed in incubator	6–9	Al-Wahaibi and Morse (2009), Pacheco et al. (2022)
Euschistus heros (Neotropical stink bug)	yellow	Not described	1.25–2 h	Double-sided sticky tape on a glass slide covered with water and wrapped with plastic film	Transfer to wet filter paper soaked with 1% Nipagen in a Petri dish	7–8	Cagliari et al. (2020)
Lygus hesperus (Western tarnished plant bug)	*Cinnabar cardinal*	Not described	1 h	Double-sided sticky tape on a coverslip	Placed in 1% agarose Petri dishes	7–9	Heu et al. (2022)
Oncopeltus fasciatus (Milkweed bug)	*white*	20 h at 28°C	2–8 h	3% *Drosophila* food-grade agarose in a mold	Placed in a sterile Petri dish, physical removal of fungus growing on agar	7	Reding and Pick, (2020)
Pyrrhocoris apterus (Linden bug)	*Cryptochrome 2* *timeless* *Period* *pigment dispersing factor* *TEFLamide*	16–19 h at 25°C	0–2 h, 0–12 h 0–12 h 2–4 h, 0–12 h 2–4 h, 0–12 h 2–4 h, 0–12 h	Double-sided sticky tape on a coverslip, covered with distilled water. Moisten embryos with water to soften chorion	Transferred to Petri dishes containing moist paper towels	7	Socha (1993), Kotwica-Rolinska et al. (2019)
Acyrthosiphon pisum (Pea aphid)	*Stylin-01 (cuticular)*	>16 h	2 h		Embryos transferred to a Petri dish containing	85	

(Continued on following page)

TABLE 4 | (*Continued*) Post-injection protocols of CRISPR/Cas9-mediated mutagenesis in Hemiptera.

Species (common name)	Gene Target	Time to Cellular Blastoderm	Time of Embryo Microinjection (h after oviposition)	Support Platform	Post-injection Treatment	Days to Hatch	References
				Embryos placed on wet filter paper on a glass slide	wet filter paper and placed in an incubator and subsequently transferred to plants		Lin et al. (2014), Le Trionnaire et al. (2019)
Bemisia tabaci (Whitefly)	*white*	Not applicable	Not applicable	Abdominal injections into adult females	Injected adult females transferred to a soybean leaflet in a Petri dish with a moist paper towels, then removed after 2 weeks and leaflet examined for gene-edited offspring	not applicable	Heu et al. (2020)

Kotwica-Rolinska et al., 2019; Le Trionnaire et al., 2019; Zhao et al., 2019; Cagliari et al., 2020; Heu et al., 2020; Reding and Pick, 2020; Klobasa et al., 2021; Xue et al., 2021; Heu et al., 2022; Pacheco et al., 2022).

Microinjection of preblastoderm embryos is the most common technique used for gene delivery in insects. Not surprisingly, this technology has dominated the hemipteran gene-editing experimental protocols. In eight hemipteran species, Cas9 protein, Cas9 mRNA, and crRNAs and tracRNAs, sgRNAs or plasmids expressing sgRNAs were directly microinjected into embryos. In most of these experiments, eggs were removed from the host plant and placed on a solid support platform. As *Lygus hesperus* eggs are usually deposited within the leaf and more difficult to excise, *L. hesperus* eggs were laid on parafilm gel packets for easy egg release for alignment for microinjections. The support varied from wet filter paper (*A. pisum*) to double-sided sticky tape on a glass slides (*N. lugens*, *Peregrinus maidis*, *Pyrrhocoris apterus*, *Euschistus heros*, and *L. hesperus*) (**Table 4**). In contrast, for *Oncopeltus fasciatus*, an agarose mold was constructed using *Drosophila* food-grade agarose. The mold held the eggs in position for sequential embryo microinjections (**Table 4**). A different approach was taken for *H. vitripennis*. Microinjection of these embryos occurred *in situ* on the leaf discs with penetration of the needle through the leaf *epidermis* and egg chorion into the embryo (**Table 4**). Finally, the approach for editing the whitefly, *B. tabaci*, was distinct as macromolecules were injected into the female abdomen (**Table 4**). This technique, called ReMOT Control (for Receptor-Mediated Ovary Transduction of Cargo) was developed as alternative to embryo microinjection (Chaverra-Rodriguez et al., 2018).

Not surprisingly, the size of insect eggs often influences the relative ease of embryo microinjection and the development of a high-efficiency gene-editing system. For some species, such as *H. vitripennis*, their larger (2.5 mm) embryos are exceptionally easy to inject (Pacheco et al., 2022). While the microinjection of the minute eggs of *B. tabaci* (~0.1 mm in length) are more challenging but technically feasible using microinjection systems where the diagonal axis of the microinjector can be precisely controlled to avoid damage to the embryonic chorion and membranes. Precision injection parameters, as well as the choice of needle, can significantly enhance injected embryo survival to adulthood. Finally, hemipteran egg chorions can be very rigid, hard to remove and for some species hard to pierce. For this reason, Kotwica-Rolinska et al. (2019) soaked *P. apterus* eggs in water to soften the chorion prior to microinjection, which significantly decreased damage to the embryo and increased egg viability and hatch.

Both quartz and borosilicate glass needles have been used successfully to deliver macromolecules in the Hemiptera (**Table 3**). Beveled quartz needles were used to penetrate the chorions of *P. maidis*, *H. vitripennis* and *L. hesperus* and the cuticle and ovaries of *B. tabaci* adult females (**Table 3**). In contrast, borosilicate needles were used for microinjections of *O. fasciatus* and *P. apterus* embryos and glass microinjection capillaries tips and glass needles were used for embryo injections in *A. pisum* and *N. lugens,* respectively (**Table 3**).

As microinjection of developing pre-blastoderm embryos predominates the hemipteran gene-editing literature (**Table 3**), it is important to note the contrasting modes of development of the hemimetabolous Hemiptera relative to the holometabolous Diptera, Lepidoptera, Hymenoptera, and Coleoptera. Holometabolous insects are the more derived developmental state having evolved from hemimetabolous ancestors some 300 MYA (Labandeira and Phillips, 1996). As both Hemiptera and Holometaboloma embryos are microinjected before cellular blastoderm, these significant developmental differences would not be predicted to impact the efficiency of CRISPR/Cas9-mediated editing of the embryo's germline cells. However, the differences in development could alter the age of the pre-blastoderm embryo and delivery site chosen for microinjection. In holometabolous species, some nuclei that contain CRISPR/Cas9-generated mutations will migrate towards the posterior pole and become germ-cell nuclei and mutations will be transmitted to future generations; while other

nuclei containing mutations will become somatic cells and will only contribute to the mutant phenotype of the G0 generation embryo, larvae or adult (Mahowald, 2001; Dearden, 2006; Nakao et al., 2006; Schröder, 2006). In contrast, in the Hemiptera, germ cells are established later in embryonic development, with the likely exception of the pea aphid *Acyrthosiphon pisum* (Chang et al., 2006; Ewen-Campen et al., 2013). Therefore, microinjections, which result in nuclei that contain mutations generated during earlier stages of embryonic development, can be delivered to both the germline and somatic cells. For this reason, one would not expect to see any significant differences in the distribution of CRISPR/Cas9-generated mutations in the germline and somatic cells in G0 larvae and adults arising from microinjected embryos from the Hemiptera. At the present time, it is unclear if the fundamental differences between holometabolous and hemimetabolous insect embryonic development alters the distribution of alleles in germline *vs*. somatic cells; this awaits rigorous testing.

Regardless of the developmental program, embryo microinjection must be performed prior to pole cell formation and cellular blastoderm, so that injected macromolecules have immediate access to the nuclei before the formation of cell membranes. In holometabolous insects, such as *D. melanogaster*, mosquitoes (*Ae. aegypti*, *An. gambiae* and *Anopheles stephensi)* and the Mediterranean fruit fly (*C. capitata)*, the time to cellular blastoderm is short, typically less than 90 min (Morris et al., 1989; Foe et al., 1993; Loukeris et al., 1995; Catteruccia et al., 2000; Grossman et al., 2001). Short times to cellular blastoderm are not strictly associated with holometabolous insects. For example, cellular blastoderm initiates at 10 h for the lepidopteran *B. mori* and 8–9.5 h for the coleopteran *T. castaneum* (Takesue et al., 1980; Handel et al., 2000). In contrast, the time to cellular blastoderm for the Hemiptera is not precisely established for all species used in gene-editing experiments. However, in the species examined to date, the duration of pre-cellular blastoderm is greater than 4 h and up to 20 h in length (**Table 4**).

Tables 2–4 provide an overview of the parameters used gene-editing experiments discussed above in the Hemiptera and the resulting efficiencies of mutagenesis. The targets and associated phenotypes will be discussed below, but it is important to note that editing success has been achieved with different experimental strategies in different hemipteran species. Therefore, consensus parameters for optimal editing in the Hemiptera are difficult to define. For example, CRISPR/Cas9 mutants have been successfully achieved by microinjecting Cas9 protein complexed with sgRNAs in most hemipteran species tested and by microinjecting Cas9 mRNAs with sgRNAs in *N. lugens* (**Table 3**). In addition, the concentrations of Cas9 protein varied markedly in these experiments ranging from 150 to 800 ng/μL. Furthermore, sgRNA concentrations varied five-fold (ranging from 80 to 400 ng/μL) and some protocols used sgRNAs complexed with Cas9, while others did not state whether they assembled the Cas9-sgRNA complexes prior to injection. Collectively, these diverse protocols indicate that variation in the quantities of the macromolecules required for editing are flexible, as there was no clear indication of an optimal concentration of either Cas9 protein, mRNA or sgRNAs across these nine species.

Other techniques have also been used to introduce nucleic acids into Hemiptera. For 15 different target genes, liposome-encapsulated dsRNAs were injected into *E. heros* second-instar nymphs (Castellanos et al., 2019). Gene silencing was observed for nine of these target genes and insect mortality exceeded 95% at 14 days post injection. While microinjection of liposomes into nymphs are an effective delivery vehicle for dsRNAs, oral delivery of liposome-encapsulated dsRNAs was less effective (Castellanos et al., 2019). Branched Amphiphilic Peptide Capsules (BAPCs) have been used as a delivery system for dsRNAs in the Hempitera and Lepidoptera. BAPCs are water-soluble nanoparticles composed of amino acids and were used to successfully deliver dsRNAs in liquid and solid diets to the pea aphid (*A. pisum)* and the red flour beetle (*T. castaneum*), respectively (Avila et al., 2018). In both insects, the gene target was the molecular chaperone BiP/GRP78, which plays a critical role in the endoplasmic reticulum's unfolded-protein stress (UPR) response. Supplementation of the insect diets with BAPC-associated dsRNAs significantly enhanced gene silencing in both insects. In addition, dsRNAs for two additional targets were delivered to *T. castaneum* using BAPC particles: *Armet*, which is important in UPR, and *vermilion*, which is involved in the ommochrome biosynthetic pathway (Avila et al., 2018). Both liposome- and BAPC-mediated delivery have the potential to deliver the CRISPR/Cas9 editing machinery to Hemiptera embryos; to date, the use of these technologies have not been rigorously tested.

Gene Editing in Hemiptera: Eye Pigmentation Mutants

As shown in **Tables 2–4**, CRISPR/Cas9-mediated gene editing has been achieved in nine hemipteran species in a relatively short period of time, as both genome projects and microinjection delivery protocols have become available. Overall, the mean frequencies of mutagenesis in the G0 generation varies from less than 1% in *B. tabaci* to as high as 100% in *L. hesperus* (**Table 2**). Eye-color pigmentation genes are the most common target genes as the ommochrome and pteridine pigment biosynthetic pathways are highly conserved across insects and mutations in these pigmentation genes provide an easily screened phenotypes (Vargas-Lowman et al., 2019). The utility of screening for eye-pigmentation phenotypes is relatively simple in insects with hemimetabolous development. For example, *H. vitripennis* and *L. hesperus* embryos with mutant eye color can be detected during the mid-late embryonic stages (Heu et al., 2022; Pacheco et al., 2022). Another advantage, especially important in the context of containment of transgenic insects, is that mutants can be identified prior to egg hatch, after which time nymphs are mobile. Plant hosts in appropriate containment cages can then be infested with nymphs with altered eye colors to determine the inheritance of the CRISPR-derived mutations.

Two eye-color genes *white (w)* and *cinnabar* (*cn*) have been used frequently for the development of CRISPR/Cas9-editing technologies in the Hemiptera (**Tables 2–4**). The most common

gene target to date is the *w* gene that encodes an ABC transporter responsible for importing precursors for both the pteridine and ommochrome pathways into cells of the developing eye. *w* has been successfully edited in all six hemipteran species in which CRISPR technology has been deployed. G0 mutagenesis frequencies are measured by the percentage of mutant G0 nymphs/the total number of G0 nymphs that were recovered from microinjection experiments of embryos or adults (**Table 2**). The mutation efficiencies were highly variable with adult injections of *B. tabaci* ranging from 0.2–2.5% and embryo injections of *H. vitripennis*, *O. fasciatus* and *L. hesperus* reaching efficiencies as high as 80, 96, and 100%, respectively (**Table 2**). Intermediate mutagenesis efficiencies were observed in *N. lugens* (27.3%) and *P. maidis* (32.4%) (**Table 2**). It is also important to note that efficiencies can vary within a species. For example, for the most efficiently edited Hemiptera to date, efficiencies can vary 1.3- to 6.6-fold for *H. vitripennis* (61.2–80.0%) and *O. fasciatus* (14.0–92.5%), respectively (**Table 2**).

While these data speak to the versatility of the *w* gene as an efficient target for establishing CRISPR/Cas9 mutagenesis in the Hemiptera, the inheritance and viability of *w* mutant homozygotes differs dramatically between species studied to date. For example, no white-eyed *O. fasciatus* individuals were observed in the G1 generation. This is despite the fact that the mosaic-eyed individuals were identified in the G0 generation and mutant *w* alleles were verified by heteroduplex analysis in heterozygous individuals from the G1 generation at a frequency of 64.6% (Reding and Pick, 2020). Furthermore, no white-eyed *O. fasciatus* mutants were recovered in the G2 generation from matings of G1 heterozygotes, despite the prediction that they should be present in 25% of the offspring (Reding and Pick, 2020). The failure to obtain homozygous *w* individuals suggests that *w* mutant homozygotes are inviable. White-eyed G0 generation embryos of *P. maidis* were also observed, however, none of these embryos hatched. Furthermore, when wild-type G0 generation adults were mated, no white-eyed progeny were recovered in the G1 generation (Klobasa et al., 2021). These data, while not as comprehensive as those obtained from *O. fasciatus*, also suggest that mutations in the *P. maidis w* gene impact viability. For *L. hesperus*, injection of *w* dsRNAs into embryos caused embryo mortality prior to visible eye formation and for this reason CRISPR/Cas9 mutagenesis was not pursued (Heu et al., 2022). Viability problems arising from mutagenesis of the *w* gene have also been observed in *D. melanogaster* and the cotton bollworm *Helicoverpa armigera* (Borycz et al., 2008; Evans et al., 2008; Khan et al., 2017; Xiao et al., 2017; Ferreiro et al., 2018; Myers et al., 2021).

In contrast, inheritance of mutant *w* alleles and eye-color phenotypes were transmitted to the G1 generations in *N. lugens* (3.2%) and *H. vitripennis* (100%), and to the G2 generation in *H. vitripennis* (100%) (Xue et al., 2018; Pacheco et al., 2022). It is also noteworthy that *H. vitripennis w* mutants displayed a phenotypic pleiotropy in both wing, eye, and ocelli color, with red pigmentation of the wing veins and cells of the forewing being absent in *w* mutants (Pacheco et al., 2022). These experiments, in conjunction with the analysis of *H. vitripennis* cn mutants, revealed that the red pigmentation patterns on *H. vitripennis* wings are due to the red pteridine pigments, rather than red melanins as previously proposed (Timmons et al., 2011).

Finally, unlike the white-eyed or mosaic-eyed mutants of the Hemiptera described above, Heu et al. (2020) reported very different putative *w* mutant phenotypes in *B. tabaci*. They used an injection mix of five sgRNAs that were complexed with a fusion protein consisting of the Cas9 protein with a short *B. tabaci* vitellogenin-binding sequence. Injections were performed in the presence or absence of the endosome escape reagent saponin. While mutants were identified with the 0 and 4 μg/ml saponin injection mixes, higher saponin concentrations (8–16 μg/ml) were toxic. Orange-eyed 4th-instar nymphs and red-eyed G0 adults were detected. The G0 insects appeared to be genetic mosaics. Inheritance of mutant alleles were inferred from phenotypes from a cross of a G1 female and her red-eyed G0 father; a non-mendelian pattern of inheritance was observed (Heu et al., 2020).

Collectively these data suggest that mutations in *the w* locus of different Hemiptera have variable phenotypes and a variable fitness costs ranging from undetectable to severe. As such, although mutations in *w* have distinct, easy to screen phenotypes, *w* is not necessarily a "risk-free" target for the development of CRISPR/Cas9 technologies in the Hemiptera.

A second eye-pigment gene, *cn*, was successfully edited using the CRISPR/Cas9 machinery in *N. lugens*, *H. vitripennis* and *L. hesperus. Cn* encodes a kynurenine hydroxylase, which, like *w*, is involved in the ommochrome biosynthetic pathway. In *D. melanogaster, cn* mutants have bright red/orange eyes (Paton and Sullivan, 1978). Wild-type *H. vitripennis* has a complex eye-pigmentation patterning with marked brown striations over a cream colored background (Pacheco et al., 2022). *H. vitripennis cn* mutants were easily identified by mosaicism in the eyes of G0 embryos, late-stage nymphs and adults (Pacheco et al., 2022). The mutation frequencies for *cn* were robust ranging from 58.9–66.7% (**Table 2**). Following pair-matings of G0 mutant adults, *cn* eye phenotypes were recovered in 100% of G1 and G2 generation individuals and their mutant alleles were verified by DNA sequence analysis. The mosaic eye-color phenotype seen in G0 generation *H. vitripennis* individuals is consistent with *cn* being a cell autonomous genetic marker in this species, as it is in *Ae. aegypti* (Pacheco et al., 2022; Sethuraman and O'Brochta, 2005).

Similar to *H. vitripennis*, *L. hesperus cn* mutants embryos and G0 nymphs and adults had bright red eyes throughout their development (Heu et al., 2022). Based on the percentage of adults with mutant eye phenotypes, *cn* mutations were generated at high frequency ranging from 40 to 100%. These mutations were heritable and were transmitted into the G3 generation.

The phenotype of G0 generation *cn* mutants of *N. lugens* was distinctly different from *H. vitripennis* and *L. hesperus.* No *N. lugens* adults with the *cn* mutant eye-color were identified in the G0 generation. However, when G0 adults were pair mated, G1 *cn* mutant adults were identified at a low frequency (3.2%) based on their bright red/orange eye color and mutant alleles were verified by DNA sequencing (**Table 2**) (Xue et al., 2018). The lack of

mosaic or eye-color phenotypes in the G0 insects suggests that the *N. lugens cn* is a non-autonomous marker. In *D. melangaster*, *cn* phenotypes are variable based on the *cn* allele ranging from non-autonomous (Beadle and Ephrussi, 1936) to autonomous (Paton and Sullivan, 1978). It was postulated that the variable *cn* phenotype could be due to insufficient amounts of 3-hydroxykyenureine in the circulating hemolymph of larvae leading to varied uptake of pigment into the eye during pupal development (Paton and Sullivan, 1978). This may occur in adults that are genetic chimeras having both wild-type and *cn* mutant proteins in their eye pigment cells. Therefore, the failure to detect the *cn* eye-color phenotypes in *N. lugens* G0 adults may indicate that *cn* could be non-autonomous in this species. Alternatively, the frequency of mutagenesis in the G0 insects may have been too low to detect mosaicism in the G0 *N. lugens* eyes due to the large amount of wild-type tissue present.

Neither Pacheco *et al.* (2022), Xue *et al.* (2018) nor Heu et al. (2022) reported fitness costs associated with mutations in *cn*. However, some mutant *cn* alleles have been associated with compromised viability in the mosquitoes *Ae. aegypti*, *An. stephensi* and *Culex quinquefascitus* (Pham et al., 2019; Bottino-Rojas et al., 2022). Gene-editing experiments in other Hemiptera are needed to resolve whether there are fitness costs associated with mutations in *cn* and whether *cn* is an autonomous or non-autonomous marker in the Hemiptera and insects from other orders.

The role of *cardinal (cd*), a second gene in ommochrome pathway, in *L. hesperus* eye color was examined by Heu et al. (2022). *Cardinal* encodes a haem peroxidase that converts 3-hydroxykynurenine into ommochromes. Like *cn*, *cd* editing occurred at high frequencies with 100% of the *surviving L. hesperus* adults being mutant. The impact of *cd* mutations were most visible in developing embryos and early instars, which showed red eyes. However, after the third instar, brown pigments gradually increased in the remaining nymphal stages and into adulthood. Although, *cd* mutant adults had redder eyes than wild-type insects, they were significantly darker than *cn* mutants. The authors suggest this is due to spontaneous oxidation of 3-hydroxykynurenine to form the brown xanthommatin (Li et al., 2017a; Figon and Casas, 2018; Zhuravlev et al., 2018).

Gene Editing in Hemiptera: Disrupting Genes Associated With Cuticle Function, Peptide Perception and the Circadian Clock

In addition to creating mutants in hemipteran genes that control eye pigmentation, mutations in ten genes that influence a wide range of functions have been pursued. These genes include loci that impact: insect cuticle pigmentation (*CSAD* and *yellow*), a putative receptor for plant viruses (*stylin-01*), two insulin receptors (*InR-1, InR-2*), a neuropeptide of unknown function (*TEFLaminde*), and the regulation of the circadian clock (*cryptochrome, timeless, period*, and *pigment-dispersing factor*) (**Tables 2–4**).

Mutations in genes that control cuticle biogenesis and color have been used as phenotypic markers for assessing the success of RNAi and CRISPR/Cas9 editing strategies in the Hemiptera. Genes that influence the cuticle were successfully mutagenized using CRISPR/Cas9-mediated gene editing in *N. lugens, E. heros,* and *A. pisum* (Le Trionnaire et al., 2019; Cagliari et al., 2020; Chen et al., 2021). In *N. lugens,* Chen et al. (2021) studied mutants in the target gene *cysteine sulfinic acid decarboxylase* (*NlCSAD*), which influences dark melanin pigment accumulation. They compared the outcomes of silencing *NlCSAD* by RNAi versus generation of *NlCSAD* null mutations. Injection of dsRNAs into 3rd-instar nymphs reduced *NlCSAD* RNAs by 65% and increased melanin levels in the cuticle. To create homozygous null *NlCSAD* mutants, *NlCSAD* was successfully edited. No visible phenotype was observed in G0 embryos or developing nymphs due to the recessive nature of the *NlCSAD* mutant alleles. Therefore, sequencing of DNAs extracted from the discarded exuvia from hatched 5th-instar G0 nymphs was used to identify putative *NlCSAD* mutants. Mutations in the *NlCSAD* gene occurred at a frequency of 9.5% and seven mutant alleles were detected (Chen et al., 2021). This low frequency may explain the absence of phenotype.

To demonstrate inheritance, two mutant G0 generation females were outcrossed with wild-type males to generate G1 generation progeny, of which 36.3% displayed a darker cuticle than wild-type controls (Chen et al., 2021). Mutant alleles were verified using 5th-instar exuvia and G1 adults with identical alleles (a 4-bp deletion in *NlCSAD*) were mated to produce the G2 generation. The G2 offspring had a genotype ratio of wild-type:heterozygous:mutant of 1:2:0.5 and phenotypic ratio of 3:0.5 (wild-type:mutant). Dark pigmentation was well correlated with *NlCSAD* allele gene dosage and *NlCSAD* RNA levels. G2 generation insects homozygous for the *NlCSAD* null allele displayed a darker cuticle color than the *NlCSAD* heterozygotes or the *CSAD*-RNAi insects. While the biochemical basis for the underrepresentation of the recessive homozygotes was not determined, homozygous *NlCSAD* null insects had reduced female fecundity and egg hatch rates, suggesting a significant fitness cost. The Chen et al. (2021) experiments emphasize two critical points. First, pursuing CRISPR/Cas9-induced null mutants in genes with unknown phenotypic ramifications, such as *NlCSAD*, is feasible. Second, the use of 5th-instar exuvia to genotype individuals of each generation provided a simple, non-invasive mechanism to identify individuals in a timely manner to allow tactical genetic crosses to be performed and, thereby, allowing mutants to be identified in the absence of strong phenotypes.

Based on studies in a number of holometabolous insects and RNAi studies with the hemipteran twin-spotted assassin bug (*Platymeris bigattatus),* the *yellow* gene is thought to be involved in the synthesis of dark melanin pigments (Zhang et al., 2019). Cuticular pigmentation was studied in hemimetabolous *E. heros* using RNAi and by creating CRISPR/Cas9-edited mutants for two target genes - *tyrosine hydrolase* and *yellow* (Cagliari et al., 2020). dsRNA silencing of the *E. heros* tyrosine hydrolase showed a reduction in pigmentation, while silencing of *yellow* did not. For this reason, CRISPR/Cas9 was used to produce null alleles to resolve the role of *yellow* in stinkbug cuticle pigmentation. *E.*

heros embryos were injected with Cas9 protein and a single *yellow* sgRNA (**Table 4**) (Cagliari et al., 2020). One G0 individual was recovered that had a 6-bp in-frame deletion in the *yellow* gene; this mutation did not disrupt the function of the *yellow* protein as this insect had wild-type cuticle pigmentation. To discover the role of *yellow* in hemimetabolous insects, *yellow* null alleles will need to be isolated in the future.

The protocols for CRISPR/Cas9 mutagenesis for the pea aphid (*A. pisum*) were established using the cuticular protein gene *stylin-01* as a target (Le Trionnaire et al., 2019). Unlike *yellow* and *NlCSAD, stylin-01* does not have a role in cuticle pigmentation. Instead, s*tylin-01* may be receptor of noncirculative plant viruses. *Stylin-01* is specifically localized to the acrostyle of the maxillary stylets, an organ that is replete with receptors for plant viruses (Webster et al., 2018). Using *stylin-01* as a target of CRISPR/Cas9 mutagenesis, Le Trionnaire et al. (2019) developed an editing pipeline for *A. pisum,* which has a life cycle that is not well adapted to routine mutagenesis strategies. Like other aphids, progeny are primarily produced by parthenogenesis resulting in genetically identical clones (Simon and Peccoud, 2018). Sexual reproduction is triggered by a shortening of the photoperiod, thereby allowing for the production of males and viviparous females, which occurs once per year. Therefore, production of developmentally synchronized males and females for the production of fertilized eggs for microinjection technologies is a major limitation. A further challenge is the fact that fertilized eggs enter a 3-month, obligate diapause prior to the emergence of the sexually derived progeny (Simon and Peccoud, 2018).

These rather daunting life cycle challenges were tackled by Le Trionnaire et al. (2019) resulting in a 7-month protocol for CRISPR/Cas9 mutagenesis of *A. pisum*. Embryos were microinjected and viable, melanized eggs transferred to plant leaves for their obligate diapause period. Sequencing of pre-diapause embryos demonstrated that 70–80% of embryos had evidence of *stylin-01* gene mutagenesis. Egg hatch occurred, but at low rates (1–11%), and 17 G0 generation foundress aphids each gave rise to a clonal colony. Sequence analyses of the *stylin-01* gene in the progeny from these clonal colonies identified six G0 foundress aphids that contained multiple *stylin-01* mutant alleles. The zygotic inheritance of these alleles has yet to be demonstrated. An additional 7 months are needed to produce the developmentally synchronized males and oviparous females required for the crosses to assess allele inheritance and the role of *stylin-01* in non-circulative virus transmission in *A. pisum*.

Two insulin receptor genes (*NlInR1* and *NlInR2*) from *N. lugens* were targets of CRISPR/Cas9 mutagenesis (**Tables 2**–**4**) (Zhao et al., 2019; Xue et al., 2021). Previous studies showed that RNAi silencing of *NlInR1* and *NlInR2* controls wing polyphenism (short versus long wings, respectively), which is important for *N. lugens* dispersal (Xu et al., 2015). The null mutants of *NlInR1* and *NlInR2* generated by CRISPR/Cas9 mutagenesis indicated that the two classes of insulin receptors have distinct roles in *N. lugens* wing development. *NlInR1* and *NlInR2* mutants have some overlapping but also distinct phenotypes indicating their different roles in growth, development and adaptation to stress. For example, homozygous *NlInR1* mutants had an early embryonic lethal phenotype, while homozygous *NlInR2* mutants did not (Zhao et al., 2019; Xue et al., 2021). Heterozygous *NlInR1* mutants grew more slowly, had reduced body mass, shorter wings, and a longer lifespan relative to wild-type control insects. These phenotypes recapitulate the impact on *InR1* mutations on *D. melanogaster* growth and development and previous RNAi silencing of the *NlInR1* (Fernandez et al., 1995; Brogiolo et al., 2001; Tatar et al., 2001; Xu et al., 2015). The severity of the *NlInR1* mutant phenotypes was correlated with the domain of the *NlInR1* locus that was mutagenized; mutations in the leucine-rich repeat domain caused more severe developmental defects than those in the furin-like Cys-rich domain. Zhao et al. (2019) also examined the *InR1*-dependent transcriptome changes in the mutant versus wild-type *N. lugens* revealing that *NlInR1* regulates genes associated with numerous cellular processes including: insulin resistance, longevity, phototransduction, cellular metabolism, endocytosis, as well as protein biosynthesis and processing. Null mutants in *NlInR2* had different phenotypes than *NlInR1* mutants providing the first insights into the distinctive roles of this understudied InR2 receptor in insects (Xue et al., 2021). In contrast to *NlInR1* mutants, *NlInR2* mutants were not lethal, displayed accelerated cell division and cell proliferation in wings (which gave rise to long wings), had defective vein patterning, prolonged developmental time, and decreased fecundity.

In addition to the insights into *NlInR1 and NlInR2* functions, Zhao et al. (2019) established important tools and principles for CRISPR/Cas9 strategies in the Hemiptera. The authors used two distinct CRISPR/Cas9 mutagenesis strategies and both produced heritable mutations in *NlInR1*. First, they microinjected of Cas9 protein with two different *NlInR1* sgRNAs. In a second strategy, they microinjected custom-designed plasmids to express the two components of CRISPR/Cas9 machinery (Cas9 and sgRNAs) within the insect embryo. One plasmid expressed a *N. lugens* codon-optimized Cas9 protein using a 2.5-kb *N. lugens vasa* (*Nlvasa)* promoter, 5′-untranslated region (UTR) and 3′-UTR (*vasa:Cas9*). In addition, two *N. lugens U6* snRNA polymerase gene promoters (*U6a* and *U6b*) were used to drive the expression of *NlInR1* sgRNAs. These experiments represent the first time endogenous promoters were used for expressing the macromolecules for CRISPR/Cas9 editing in hemipteran embryos.

Zhao et al. (2019) also systematically analyzed the mutagenesis efficiencies using microinjected Cas9 protein and sgRNAs versus the *vasa:Cas9* and *U6:sgRNA* plasmids for gene editing. Microinjections using the *vasa:Cas9* plasmid instead of Cas9 protein increased the survival to adulthood by seven-fold perhaps due to the toxicity of the Cas9 protein (Zhao et al., 2019). Amplicon sequencing of G0 and G1 generations adults determined gene-editing frequencies. The frequency of inherited mutations in the G1 generation was higher in embryos microinjected with the Cas9 protein versus the *vasa:Cas9* plasmid. These data suggested that the immediate presence of Cas9 activity in embryos may be critical for efficient germline mutagenesis. In balancing the two findings, the authors concluded that the use of the plasmid DNAs as source of Cas9 was the optimal and more economical method for gene editing in

N. lugens. The *vasa:Cas9* gene may be an important tool for increasing the efficiency of CRISPR-mediated editing in the future. If similar to other systems, integration of *vasa:Cas9* into the *N. lugens* genome by homology-directed repair or transposon mutagenesis will result in an increase in CRISPR/Cas9 editing to boost efficiencies of both knock-in and knock-out mutagenesis (Sebo et al., 2014).

A neuropeptide of unknown function (TEFLamide) and four genes associated with the circadian rhythm were targeted in *P. apterus* (**Tables 2–4**) (Kotwica-Rolinska et al., 2019). Microinjection of Cas9 proteins and sgRNAs into *P. apterus* embryos was performed. Putative gene edits in G0 and G1 generation individuals were initially identified by heteroduplex analysis and then confirmed by sequencing. The significance of these data is several-fold. First, an effective gene-editing protocol in *P. apterus* for five genes, whose mutations did not provide a clear morphological phenotypes to enable screening, was achieved. Second, the frequency of gene editing in G0 generation individuals, measured by heteroduplex assays, was extensive, ranging from 35.4–83.4%. The optimization of these relatively inexpensive heteroduplex assays to rapidly detect mutagenesis at the target sites served as their "phenotypic" screen. Third, the mutagenesis rates in embryos microinjected with *Cas9* mRNA vs. protein were compared. While G1 mutations were detected in G1 heterozygous individuals generated by *Cas9* protein injections, no mutants were recovered when *Cas9* mRNA was microinjected. However, this needs to be placed in context, as the total number of gene-editing events recovered was small (six gene edits from five G0 parents after 1,280 embryo injections) (Kotwica-Rolinska et al., 2019). In addition, these results contrasted those of Xue et al. (2018) in *N. lugens*, who detected *w* and cn mutants after microinjection of *Cas9* RNAs (97 gene edits from eight G0 parents and 1,064 microinjections); however, they did not directly compare editing efficiencies using microinjected *Cas9* protein vs. *Cas9* mRNA.

Kotwica-Rolinska et al. (2019) tested the efficacy of sgRNAs versus a two-component RNA system that used crRNAs and tracrRNAs using the *pigment-dispersing factor* (*pdf*) gene as the target. Both sgRNAs and crRNA/tracrRNAs generated G1 mutants. There was variation in replicate experiments and, given the small number of embryos injected, it is not yet possible to conclude if sgRNAs or the two-component system was more efficient. However, the reduced cost and availability of sgRNAs makes sgRNA use preferred (Kotwica-Rolinska et al., 2019).

Kotwica-Rolinska et al. (2019) also assessed if the age of injected embryos influenced gene-editing frequencies using Cas9 protein and *pdf* crRNA/tracrRNA. Cellular blastoderm occurs at 16–19 h following oviposition in *P. apterus* providing a large window of opportunity for microinjections (Socha, 1993). Interestingly, G1 mutants were detected at a frequency of 24.1% in embryos after an overnight oviposition (0-to-12 h-old embryos), while the frequency was 1.7% for embryos from a 2-to-4 h oviposition period (Kotwica-Rolinska et al., 2019). As these are the only data examining the timing of embryo microinjections in the Hemiptera and, despite the fact that a small number of mutants were generated per G0 adult, we may be able to conclude that, in those species with long pre-blastoderm periods, microinjection at later times may generate higher frequencies of gene editing. It would be interesting to further examine this using morphological makers that provide easier identification of gene-edited alleles.

Understanding the accuracy of CRISPR/Cas9 editing is critical for evaluating the biological impact of mutations generated by this system. In the Hemiptera, generation of off-target mutations has been examined in only two studies (Xue et al., 2018; Pacheco et al., 2022). Given the exceptionally high rates of CRISPR/Cas9-editing in *H. vitripennis* (**Table 2**), Pacheco et al. (2022) analyzed *w* and *cn* sgRNA specificity *in vivo*. Cas-OFFinder was used to identify potential off-target sites (Bae et al., 2014). Off-target candidates within transcribed regions of genes that had an exact match to the PAM site and all 7 bp in the seed region adjacent to the PAM site were candidate off-targets. The cutting frequency determination score was used to rank potential off-targets for their likelihood of being cleaved by the Cas9 protein (Doench et al., 2016). Finally, off-targets with nucleotide polymorphisms or bulges furthest away from the seed region were chosen for analysis, as they would be the most likely off-target sites. Four to five off-target sites for two *w* and one *cn* sgRNAs were chosen for analysis. Off-target mutagenesis was assessed in genomic DNA from four *w* G0 and six *cn* G0 females. Of the 11 amplicon libraries analyzed, the mean percentage of reads mapping to the off-target sites ranged from 0 to 0.95%; however, one *w* sgRNA had a mutation rate of 5.04%. These data indicated that off-target editing did not occur or occurred infrequently in G0 embryos. It is also important to note that as off-target mutations were assessed in G0 generation insects, their inheritance was not determined. The *H. vitripennis* off-target frequencies compared favorably to data from *An. gambiae* when off-target effects on a gene-drive strategy were assessed (Garrood et al., 2021).

Using the sgRNACas9 algorithm, Xue et al. (2018) identified a total of 54 and 100 putative off-target sites with 1–5 bp mismatches for the *N. lugens cn* and *w*, respectively. They determined mutations for four *cn* and three *w* off-target sites in three G1 *cn* and three G1 *w* mutants, respectively. Each of these off-target sites had five mismatched base pairs, with mismatches located at different proximities to the predicted Cas9 cut site. No evidence of off-target mutagenesis was detected. Collectively, these data from *H. vitripennis* and *N. lugens* indicate that off-target mutagenesis occurs infrequently at sites highly related to the sgRNA target site. The ability to accurately identify off-target sequences for sgRNAs is critical for successful and precise gene editing, both for unequivocally determining gene function and for genetic control strategies in which the genotype of a strain for release into the field needs to be known with certainty. These will require reference genome assemblies of high levels of accuracy to be available, as well as standardized criteria for objectively evaluating how to best identify off-target sites and how then to design mutagenesis experiments within them. Alternatively, or in addition, phenotypes from independently-generated mutant lines combined with several generations of out-crossing can also reduce or eliminate the effect of any off-target mutagenesis.

CRISPR/Cas9-mediated gene editing has not yet been used to generate gene insertions for gain-of-function mutants in

Hemiptera. These "knock-in" mutations would be expected to occur at lower frequencies than the knock-out gene-editing frequencies reported based on studies in other insects (**Table 2**) (Gratz et al., 2014; Wang et al., 2017; Kandul et al., 2020). However, given the rapid extension of CRISPR/Cas9 technology into the Hemiptera, it is reasonable to expect that knock-in mutagenesis will soon be achieved.

Somatic Assay Platforms: Hemiptera Cell Culture

Insect cell culture provide an opportunity to enhance and accelerate the experimental design of gene editing *in vivo* by testing parameters for efficient CRISPR/Cas9-mediated gene editing by identifying efficient sgRNAs and any potential off-target effects associated with the CRISPR/Cas9 mutagenesis. In addition, cell cultures allow the rapid testing of the ability of putative regulatory sequences (e.g, promoters and untranslated regions) to drive gene expression. The ability to use cell lines to optimize CRISPR/Cas9 mutagenesis in non-hemipteran insects is established. These studies have assessed: the ability of sgRNAs and Cas9 to cut target gene sequences, tissue-specific CRISPR, and optimization of Cas13 cutting (Meltzer et al., 2019; Viswanatha et al., 2019; Huynh et al., 2020; Trivedi et al., 2020).

Despite the fact that cell culture lines from 20 hemipteran species have been described and used primarily for use in plant-virus-vector interactions (**Supplementary Table S2**) (Boyapalle et al., 2007; Marutani-Hert et al., 2009; Jia et al., 2012; Wang et al., 2018), no CRISPR experiments have been performed in hemipteran cell cultures. However, an *H. vitripennis* cell culture has been used to assess the efficiency of RNAi after the delivery of *actin* dsRNAs via lipotransfection (Rosa et al., 2010). In addition, the activities of a small number of hemipteran promoters have been assessed in hemipteran, lepidopteran and dipteran cell culture lines (see Hemipteran promoters below) (Qian et al., 2016; Zhao et al., 2019).

Finally, insect cell cultures have been used to assess the activity of a small number of proteins in insecticide resistance. For example, *Sf9* cells from *S. frugiperda* were used to understand the roles of calcium-binding proteins and calmodulin in insecticide resistance in *B. tabaci* and G protein-coupled receptors in *H. halys* (Guo et al., 2019; Ahn et al., 2020; Guo et al., 2021). *D. melanogaster* cell lines were used to understand the impact of a mutation in the *Laodelphax striatellus* GABA receptor activity on resistance to insecticides (Nakao et al., 2011).

Hemipteran Promoters

Germline and constitutive promoters are essential elements for any genetic toolbox used for the genetic manipulation of insects. Promoters can drive the expression of Cas9 *in vivo* to enhance CRISPR/Cas9 mutagenesis or fluorescent reporter genes to track transgene insertion events by CRISPR/Cas or transposons. To date, few hemipteran promoters have been isolated and characterized and no promoters from other insect orders have been tested in hemipteran cells culture or *in vivo*. Constitutive, germline-specific and tissue-specific promoters from target hemipteran species need be isolated and characterized. The hemipteran genetic toolbox requires RNA polymerase II and III promoters to drive the expression of mRNAs and small RNAs, respectively. Currently, the only tested endogenous hemipteran promoters are from planthopper species (Qian et al., 2016; Zhao et al., 2019).

U6 promoters use RNA polymerase III to naturally direct the synthesis of small, highly abundant non-coding RNA transcripts in eukaryotes. *U6* promoters and their transcriptional terminators have been used to drive expression of sgRNA genes and small dsRNAs for gene-silencing strategies in planthoppers (Zhao et al., 2019). Two *U6* promoters, *U6a* (431 bp) and *U6b* (455 bp), were used in *N. lugens* CRISPR/Cas9 experiments. Microinjections using the *U6:sgRNA* plasmids showed that the *U6b* promoter outperformed the *U6a* promoter *in vivo* when the editing of the *N. lugens NlInR1* target gene was assessed (Zhao et al., 2019). At present a systematic evaluation of the benefits of using *U6:sgRNA* plasmids versus purified sgRNAs in embryo microinjection experiments has not been performed.

Constitutive polymerase II promoters from *N. lugens* and the green rice leafhopper (*Nephotettix cincticeps*) were identified, isolated, and tested (Qian et al., 2016). Three *N. lugens actin* genes (*Nl_act1-3*), two *N. cincticeps actin* genes (*Nc_act1-2*) and one *N. lugens α-tubulin* gene (*Nl_α-Tub*) were tested for their ability to drive the expression of a green fluorescent reporter protein (GFP) in insect cell culture lines. Each construct had ~2-kb of promoter sequence and its associated 5′- and 3′-UTRs. Vectors with *promoter:GFP* constructs were transfected into S2 cells from *D. melanogaster*, Sf9 cells from *S. frugiperda* or BmN cells from *B. mori*. Only *Nl_act1-3, Nc-act1* and *Nl_α-Tub* were active (Qian et al., 2016). While *Nl_act3* and *Nc_act1* promoters were active all three insect cell lines, *Nl_α-Tub* was only active in S2 cells, albeit at very high levels (Qian et al., 2016). However, quite surprisingly, none of these promoters were active the *N. lugens* cell line tested (Qian et al., 2016).

These experiments show the value of using insect cell culture to systematically test promoter activity by following expression of a fluorescent marker. However, these data also serve as a cautionary note; while some constitutive promoters are active in multiple cell lines, others may have cell line-specific patterns of usage. Current cell lines cannot provide information about the tissue-specific usage of promoters; therefore, tissue specificity or constitutive expression is often inferred from conserved motifs within promoters, expression data, or data from orthologs of other insects in the literature. In this context, both the *Nl_act3* and *Nl_act2* group are most highly related to the muscle-specific actins from other insects (Qian et al., 2016). The activities of the planthopper *actin* and α-*tubulin* promoters *in vivo* await characterization by transient assays in microinjected embryos or after transgene integration into an insect genome.

Most germline-specific promoters that have been used extensively in insect biotechnology are derived from holometabolous insects (Adelman et al., 2007; Li et al., 2017b). There are fundamental differences in the mechanisms for germline formation in holometabolous and hemimetabolous insects, with the possible exception of *A. pisum*. Therefore, it is possible that a different set of germline promoters will be needed for the hemimetabolous Hemiptera. Based on sequence

identity to the germline *vasa* of *D. melanogaster,* the *Nlvasa* gene was identified and its promoter was used to generate *NlInR1* and *NlInR2* mutants in *N. lugens* (Zhao et al., 2019; Xue et al., 2021). The ~2-kb *vasa* promoter is the first RNA polymerase II-dependent putative tissue/cell-specific promoter has been used in the Hemiptera. Prior to the use of a plasmid expressing *vasa:Cas9* gene in embryo microinjection experiments, its ability to drive the expression of luciferase in *D. melanogaster* S2 cell cultures was demonstrated (Zhao et al., 2019).

While Zhao et al. (2019) demonstrated the activity of *vasa* in developing embryos and its ability to express sufficient quantities of Cas9 for gene editing in *N. lugens*, it is unclear if *vasa* is actually expressed in *N. lugens* germline cells. Given the differences in hemimetabolous and holometabolous germline development, it is possible that the *N. lugens vasa* will be expressed in a different manner than its *D. melanogaster* or mosquito orthologs. Germline establishment occurs by one of two mechanisms - maternal provision or zygotic induction (Extavour and Akam, 2003). Maternal provision is active in holometabolous insects, such as *D. melanogaster*. In this scenario, germplasm is generated during oogenesis and the germ cells form early in during embryogenesis at a specific site within the embryo. In contrast, zygotic induction requires the activation of the zygote genome before the germline is established and, accordingly, occurs later during embryonic development. Zygotic induction is the ancestral state of germline determination (Extavour and Akam, 2003).

The *Drosophila oskar* gene encodes a germ granule protein that is synthesized at the posterior pole of the oocyte. *Oskar* initiates germplasm assembly and germ cell determination; this gene appears to have been lost in the Hemiptera (Ephrussi and Lehmann, 1992; Lynch et al., 2011; Quan and Lynch, 2016; Blondel et al., 2021). For this reason, it is expected that there should be fundamental differences in the transcriptomes of embryos during oogenesis and early embryogenesis. This is supported from the analysis of *O. fasciatus* transcriptomes, which showed that 19 genes (orthologous to genes associated with germ cell formation in *D. melanogaster)* were expressed uniformly during these early embryonic stages (Ewen-Campen et al., 2013). This suggests that, at least in *O. fasciatus*, there is no maternal provision of the germplasm. In addition, the *O. fasciatus vasa* gene was not associated with germ cell formation but was required for spermatogenesis. In *N. lugens, Nlvasa:Cas9* and sgRNAs were active in the germ line and generated CRISPR/Cas9 mediated mutations that were inherited.

In addition, there are significant differences in expression programs of embryonic pair-rule genes. Pair-rule genes are expressed with different temporal and spatial patterns during embryogenesis in *D. melanogaster* and the hemipterans *O. fasciatus* and *Murgantia histrionica* (the harlequin bug) (Auman and Chipman, 2018; Reding et al., 2019; Hernandez et al., 2020). These surprising differences within the Hemiptera indicate that expression profiles of candidate germline-specific promoters from the Hemiptera should be characterized prior to use in CRISPR-based experiments that seek to specifically direct expression of transgenes to the male or female germline.

The rapid and successful development of CRISPR/Cas9-mediated knock-out mutagenesis in Hemiptera since 2018 increases the demand for tissue/cell-specific and constitutive promoters, which will be important for developing gain-of-function mutagenesis. Additional germline-specific promoters will need to be identified, as they are important for generating insect lines that express Cas9 in germline cells to enhance the frequency of gene insertion events *via* Cas9-mediated mutagenesis. Containment of Cas9 activity within the germline or early embryonic stages will be advantageous for reducing the number of off-target mutations, which could potentially confound any genetic strategy using CRISPR-mediated mutagenesis. Furthermore, identification of robust constitutive promoters that can drive fluorescent reporter gene expression *in vivo* will be a useful addition to the hemipteran genetic toolbox. Chimeric genes utilizing these promoters can be used as reporters to monitor gene integration *via* by CRISPR/Cas or transposon technologies. The *in vivo* activities of candidate promoters can be quickly screened in transient somatic assays in microinjected embryos, nymphs, or adults by their fluorescence. Alternatively, screens can occur in cell culture lines, with the caveat that not all "constitutive" promoters from the Hemiptera are active in all cell lines (Qian et al., 2016).

Transposable Elements

Transposable elements are effective genetic tools for introducing and integrating exogenous DNA into the germline of insects. Until the extension of CRISPR/Cas9 knock-in technology using homology-directed repair into *D. melanogaster,* transposon-mediated gene integration was the only effective way generating gain-of-function mutants in insects (Gratz et al., 2014). Today, transposable elements are routinely used to integrate the CRISPR/Cas9 machinery, other transgenes, or reporter genes into insect genomes (Galizi et al., 2016; Li et al., 2017b; Meccariello et al., 2021). Insect lines that express Cas9 from their genomes are an important genetic resource used to increase the frequency of gain-of-function or loss-of-function mutations and are now utilized to enable the rapid development, testing and implementation of genetic control strategies for insect control. Insect transposable elements, such as *piggyBac* from the cabbage looper (*Trichoplusia ni)* and *Mos1* from *Drosophila mauritiana*, have wide host ranges and may be candidates for transposon-mediated mutagenesis in the Hemiptera. To date, transposon-mediated genetic transformation has yet to reported in the Hemiptera. However, the genomes of Hemiptera are replete with transposons that could enable this technology.

A. pisum, *O. fasciatus*, *H. halys*, *P. venusta*, *H. vitripennis*, and C. *lectularius* genomes have large numbers of *mariner-Tc1*, *Helitron*, *hAT*, and *piggyBac* transposons, with the *mariner-Tc1* superfamily being the most abundant (Peccoud et al., 2017). While an actively mobile transposon has not yet been identified in the Hemiptera, two full-length and potentially active *mariner*-like transposable elements (*Btmar1.1* and *Btmar2.1*) were recently identified in the *B. tabaci* genome (Zidi et al., 2021). Compared to *mariner* elements from other insect species, the *B. tabaci mariner* elements have longer terminal-inverted repeats. In the future, it will be interesting to understand if

Btmar1.1 and *Btmar2.1* can mobilize other *B. tabaci mariner* elements, if full-length *mariners* from other insect species can mobilize *B. tabaci mariner* elements, and finally if these mobile elements can function in other hemipteran species. The increasing numbers of well-annotated hemipteran genomes should facilitate these endeavors, as well as identifying if Hemiptera contain any novel active mobile elements from other transposable element superfamilies. This is quite feasible since an analysis of the abundance and diversity of transposable elements in 42 different arthropod species revealed that the hemipteran genomes have the greatest diversity of transposable-element superfamilies (Petersen et al., 2019). On average, hemipteran genomes contained a mean of 55.7 transposon superfamilies, compared to 48.5 superfamilies in the dipteran species (Petersen et al., 2019).

Insects with loss-of-function mutant alleles in eye-pigmentation genes may also have utility in development of efficient transposon mutagenesis strategies, as changes in eye color are easy to screen and can be detected in embryos, nymphs and adults (see section entitled Gene Editing in Hemiptera–Eye pigmentation mutants). This proposed strategy must exclude any Hemiptera in which null mutations in eye-color genes are accompanied by significant fitness costs or lethality. However, it is our experience that for some Hemiptera, such as *H. vitripennis*, *w* or *cn* mutant strains can be easily maintained for greater than six generations (Pacheco, Walling and Atkinson, unpublished). Such strains could serve as the parental genotypes for transposon-mediated gene insertion. Upon integration into the parental genome, transposons can deliver their cargo (a wild-type eye color gene under the control of constitutive promoter plus a gene of interest) to random sites in an hemipteran genome, thereby complementing the null *w* or *cn* mutation and restoring the wild-type eye color. This should provide a rapid and sensitive screen for transposable element insertion. As genomic safe-harbors (genome sites that allow high levels of transgene expression) are not yet characterized in the Hemiptera and most other insects, the use of eye pigmentation markers should rapidly advance the ability to generate and detect gain-of-function mutations. Alternatively, the transposable element's cargo could be a fluorescent protein gene under the control of a constitutive or germline promoter; again providing a rapid and reliable screen for transgene insertion. Finally, as this technology develops, hemipteran lines that express a transposase gene under the control of an inducible promoter (i.e., a heat-shock promoter) would add additional ease to deploying transposon-mediated mutagenesis in the Hemiptera.

Future Tools and Their Impact on Genetic Control Strategies

With the feasibility of editing hemipteran pests now established, we are entering a new phase of technology development focusing on the critical tools for deploying CRISPR/Cas9 editing. We will be able to interrogate developmental pathways in the Hemiptera and the biochemical and genetic pathways that underpin the interactions of hemipteran insects with their host plant species and the pathogens that they transmit. As we have seen in *N. lugens*, one of the most advanced hemipteran systems using CRISPR/Cas9 technology, new insights to insect gene function and evolution have already been revealed (Zhao et al., 2019; Xue et al., 2021). Furthermore, mutating *H. vitripennis' w* and *cn* provided unanticipated insights into the origins of red pigments in forewings and the complex pigmentation patterns of *H. vitripennis* eyes. Finally, new genetic, chemical or behavioral solutions for the control of these insects will likely emerge.

To transition to development of genetic control mechanisms, there is now a pressing need for the development of efficient methods for creating gain-of-function mutants *via* gene integration. One limitation to deploying these technologies is the fact that genomic safe harbors for insect genomes are just beginning to emerge (Miyata et al., 2022). Genomic safe harbors are genome target sites that allow for the stable expression of transgenes without phenotypic consequences for the organism. For some Hemiptera, potential genomic safe harbors have already been identified such as *H. vitripennis' w* and *cn* loci and *L. hesperus' cd* and *cn* loci (Heu et al., 2022; Pacheco et al., 2022). For most Hemiptera, genome safe harbors will need to be identified. For this reason, transposon-mediated gene delivery is likely to come to the forefront as "random" sites of integration are used to identify genomic safe harbors that promote robust transgene expression. Subsequently, the presence or absence of detrimental impacts on hemipteran function can be assessed to identify the optimal sites for the integration of transgenes.

Once genome safe harbors for the Hemiptera are identified, the gain-of-function strategies for genetic control can be deployed leveraging the target-site gene integration methods afforded by CRISPR/Cas9 technology. Gene insertion cassettes can be introduced using CRISPR/Cas9-mediated homologous DNA repair or CRISPaint that uses nonhomologous end-joining (Gratz et al., 2014; Bosch et al., 2020). However, based on other insect systems where these technologies are established, these events are likely to be 10- to 100-fold less frequent than loss-of-function editing. For these reasons, strong tissue-specific and constitutive promoters will be needed drive robust visual reporters allowing for the development of time-savings, unambiguous phenotypic screens. In addition, the development of these technologies will also add a needed layer of rigor to current CRISPR/Cas9 genetic strategies that have been deployed in insects–the ability to prove unambiguously that a CRISPR/Cas9-derived mutation is the cause of a phenotype. Essential for this is the ability to complement a mutant and restore gene function; this is critically important given the fact that, while occurring at low to undetectable levels, off-target mutations can occur in CRISPR/Cas9-edited organisms. Alternatively, whole genome sequencing or extensive backcrossing will be needed to verify segregation of a phenotype with the mutation. Finally, another useful tool for genetic control will be the development of site-specific

recombinase systems for the Hemiptera. These technologies will allow precise integration of genes into genomic safe harbors without risk of off-target site mutagenesis. In addition, these methods would allow the facile movement of gene cassettes in and out of the genomes of Hemiptera (Schetelig and Handler, 2013; Häcker et al., 2017; Schetelig et al., 2019).

With an improved set of genetic tools for the Hemiptera, contemporary genetic approaches, such as gene drive for population replacement or population elimination, may also now be pursued. However, a fundamental question exists - can gene drive strategies be developed and deployed in Hemiptera? As mentioned in the Introduction to this review, many Hemiptera lack the biological traits that easily enable genetics-based population control strategies, such as SIT. However, some Hemiptera have been considered excellent targets for genetic control. In assessing the potential use of CRISPR/Cas9 technologies for agricultural pest control, Scott et al. (2018) pointed to five potential target species: the new world screwworm fly (*Cochliomyia homnivorax*), the spotted wing *Drosophila* (*D. suzukii*), the diamondback moth (*P. xylostella*), the red flour beetle (*T. castanuem*), and the whitefly (*B. tabaci*). All of these species have high fecundity, short life cycles and are invasive species with global impacts on agriculture. We might argue that many hemipteran pests have these and other attributes that make these insects priorities for genetic control strategies.

The pros and cons of gene drive deployment as a genetic control in agriculture has been reviewed (Courtier-Orgogozo et al., 2017; Baltzegar et al., 2018; Legros et al., 2021). Furthermore, the dynamics of various gene-drive strategies for replacement or elimination of pest populations have modeled diploid pests with XY sex determination (Galizi et al., 2016; Hammond et al., 2016). However, some Hemiptera are haplodiploid and others lack a Y chromosome (XO) and these fundamental genetic differences may influence the efficacy of gene drive in these Hemiptera (Pal and Vicoso, 2015; Blackmon et al., 2017; Meccariello et al., 2021). Indeed, our models probing the efficiency of gene drive in *B. tabaci*, which has haplodiploid method of sex determination, indicate that if the alleles to be driven through a population have a small fitness cost or have a fitness advantage, the efficiency of the drive in haplodiploids is not appreciably different from drive systems in diploids (Li J. et al., 2020).

It may also be possible to develop gene-drive technologies for the Hemiptera that have longer life cycles, are not as fecund as *B. tabaci* and may be more problematic to rear. As noted by Legros et al. (2021), one advantage of agricultural targets is that their environment is managed, accessible and relatively small, unlike environments in native ecologies that are the geographic venues for gene edited-based mosquito gene-drive projects. An additional consideration to deployment of genetic control is the impact of a pest on its agricultural hosts in a specific region and stakeholder perceptions of gene-drive impacts. For example, for some host plant-pest interactions, feeding damage alone can suppress yields and require intervention; at these sites, genetic-control mechanisms to eradicate populations would be preferred. For other pests, losses are primarily associated with their ability to vector pathogens. In regions that are pathogen free, control strategies could focus on population replacement strategies with a benign gene-edited strain that cannot transmit the pathogen. Stakeholders may view population replacement with a benign strain a feasible strategy provided that gene-drive models are integrated with economic models and there is demonstrated cost savings to the stakeholder. A demonstrable and continual economic benefit for the stakeholder may mean that a program that takes several seasons to deploy and achieve its goal would be acceptable.

If a long-term view for control of a pest population can be embraced, low-threshold drives (release of modest number of gene-editing insects annually) could be deployed. Low-threshold drives maybe preferred since the release of large numbers of an edited strain has the potential to increase crop damage in the immediate term, which is not an economically desirable outcome. The use of low-threshold gene drives also reduces the need to have large mass-rearing facilities, which may not be feasible for some hemipteran pests. Finally, by using a private allele strategy in CRISPR/Cas-mediated gene drives, the control strategies can be contained and the drive's impact can be limited to a specific target population (Willis and Burt, 2021). This specificity can be enabled by the precision editing controlled by Cas endonucleases and the choice of sgRNA sites that are present in a target population and absent non-target populations of the same species.

Based on strategies being developed for mosquitoes and the Mediterranean fruit fly, some of these control strategies may require manipulation of sex ratios (Kyrou et al., 2018; Meccariello et al., 2021). This will require a much greater understanding of the mechanism of sex determination in the Hemiptera. Mechanistically, the gene networks that control hemipteran sex determination must be revealed to assess if common or unique solutions to sex determination are used. To this end, orthologs of the *transformer*, *transformer2* and *doublesex* genes have been cloned and analyzed from *B. tabaci* (*tra, tra2, dsx*), *N. lugens* (*dsx*) and *R. prolixus* (*tra*, *dsx*); in addition, a novel gene that is a feminizing switch called *female determiner (Nlfmd*) has been identified as potential regulator of *dsx* in *N. lugens* (Xie et al., 2014; Guo et al., 2018; Zhuo et al., 2018; Wexler et al., 2019; Zhuo et al., 2021). CRISPR/Cas9-gene editing technology will provide a precise tool with which to accelerate our understanding of the mechanism of sex determination in Hemiptera and so provide opportunities for manipulating the sex ratio of target populations.

Identifying the reproductive biology and mating pattern of target species is mandatory for any successful gene-drive strategy. All gene drives depend on mating and fertilization between transgenic and wild-type individuals to bias the inheritance of a specific gene from one generation to the next (Dhole et al., 2020). Non-random mating can influence the spread of the drive, while inbreeding and multiple matings are of particular concern (Drury et al., 2017; Zentner and Wade, 2017; Champer et al., 2021).

Understanding a pest's mating system is also essential to determine the dynamics of population growth for the target

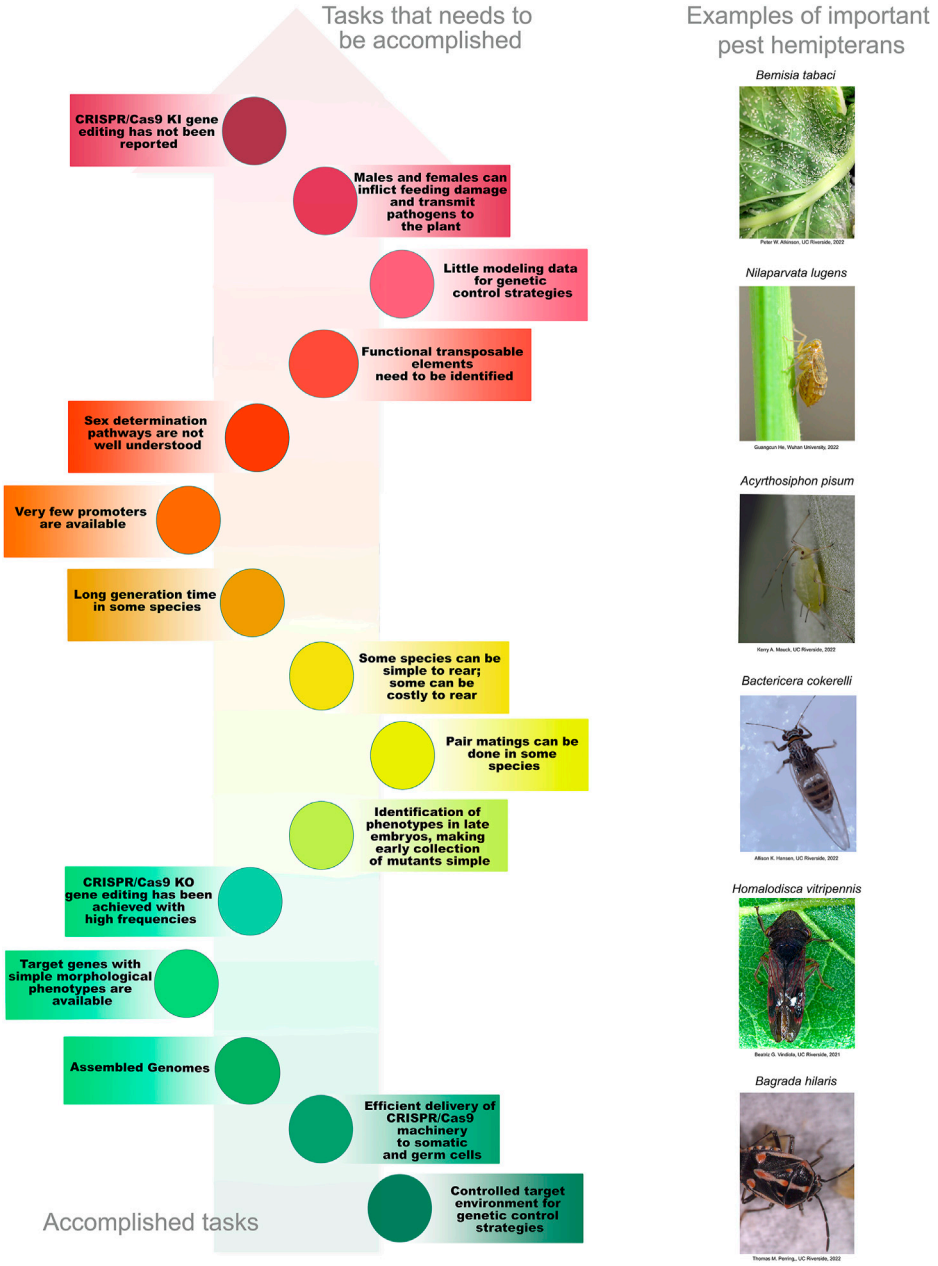

FIGURE 5 | The progress made in establishing genetic technologies in the Hemiptera with green circles and panels depicting tasks that have been accomplished, pale green to tan circles and panels depicting relevant biological traits among Hemiptera that may affect our ability to efficiently extend genetic technologies to some species, and orange to dark red circles and panels depicting tasks that still need to be accomplished.

organism. For gene drives that promote male scarcity (Y-shredder) or polygynous populations (where males mate with multiple females) and only males are released, a much stronger bias in the sex ratio is required to achieve the same level of suppression than monogamous population strategies (Prowse et al., 2019). In addition, in sperm/male-killing strategies, male infertility can modulate female behavior by increasing female remating after ineffective matings (Kraaijeveld and Chapman, 2004; Charlat et al., 2007; Price et al., 2008; Iyengar and Reeve, 2010; Sutter et al., 2019; Sutter et al., 2021). Moreover, polyandrous mating systems, where females mate with multiple males, can limit the drive spread by reducing the probability that the egg will be fertilized by a drive-carrying sperm (Manser et al., 2020; Sutter et al., 2021). Multiple matings can also increase sperm competition (Iyengar and Reeve, 2010; Greenway et al., 2022). If the gene drive-carrying males have a reduction in sperm number and quality, sperm competition can hamper the

reproductive ability of these animals and consequently suppress gene drive (Manser et al., 2017; Dhole et al., 2020; Manser et al., 2020; Price et al., 2020). These considerations are all pertinent to the development of future gene-drive strategies for the Hemiptera. Polygamy has been reported in several economically important Hemiptera such as stink bug, psyllid, mealybug, and glassy-winged sharpshooter (Mau and Mitchell, 1978; Seabra et al., 2013; Lubanga et al., 2018; Moura and Gonzaga, 2019; Silva et al., 2019; Cingolani et al., 2020; Gordon and Krugner, 2021).

Hemiptera present opportunities for genetic control in local, controlled, and managed environments. Gene-drive strategies must be adapted to the sex-determination mechanisms, mating propensities, life cycle features, and regional environmental conditions. Models to predict efficacy of a gene-drive strategy, as well as impacts on non-target organisms and the possible evolution of gene-drive immunity within the target population, will need to be developed. These models must also assess both short-term and long-term economic impacts to a region for stakeholders to embrace these longer-term solutions to hemipteran pest control.

Currently, there is indication of public support for gene drive applications for the control of insect pests of US agriculture (Jones et al., 2019). This study explored reaction to gene drive research in two insects of agriculture, the spotted wing *Drosophila* (*Drosophila suzukii*) and the hemipteran Asian citrus psyllid (*Diaphorina citri*). Public support was influenced by whether non-native or native species are targeted, whether the gene drive will be contained, and the entity conducting the experiments. The cost-effectiveness of the program and the speed of the gene-drive spread were of less concern than any impacts on human health and any adverse ecological impacts from removal of the target pest species (Jones et al., 2019). While other contemporary genetic technologies for inset pest control were not part of this survey, the authors hypothesized that similar public support would likely be extended to these, with the same qualifications (Jones et al., 2019). For these reasons, selected hemipteran pests may present an attractive platform for CRISPR/Cas-based genetic-control strategies. For example, a target pest species that was invasive, was confined to a managed environment in a specific geographical region isolated from related native species could present an opportunity for a controlled genetic-based elimination or replacement strategy that had economic parameters acceptable to the relevant industry and ecological parameters acceptable to other stakeholders.

CONCLUSION

With current and emerging hemipteran genomes and the establishment of the CRISPR/Cas9 methods for creating mutations in target genes in hand, the hemipteran community is poised for rapid advances in the mutational analysis of these non-model insect species. Pipelines are now developed for nine hemipteran species. The protocols for the microinjection of Hemiptera embryos have now been developed and tuned to the unique biological characteristics of each insect. While protocols for some Hemiptera result in consistently high mutational frequencies, other protocols will need enhancements for larger scale deployment (**Tables 2**, **3**). Collectively, the innovations that have been successfully deployed in the Hemiptera are remarkable. As reported in **Tables 2** and **3**, small advances like finding optimal rearing conditions to prevent *H. vitripennis* from entering its winter diapause (Pacheco et al., 2022), creation of molds to cradle fragile eggs for microinjection (*O. fasciatus*) (Reding and Pick, 2020), water soaking to soften tough chorions to facilitate microinjection and promote egg hatch (*P. apterus*) (Kotwica-Rolinska et al., 2019), use of gel-filled parafilm sachets for egg deposition (*L. hesperus*) (Heu et al., 2022), the development of cost effective methods to screen for allele inheritance non-invasively by extracting DNAs from nymph exuvia thereby demonstrating the ability to rapidly genotype individuals to identify edited G0 progeny without visible phenotypes (*N. lugens*) (Zhao et al., 2019), and exploration of the ReMOT system for the delivery of the CRISPR/CAS9 mutagenesis machinery (*B. tabaci*) (Heu et al., 2020), are examples of system innovations, some with high return. In addition, the persistence of hemipteran researchers to compress the daunting 12-month sexual mating cycle of the pea aphid to 7 months in controlled environments has allowed for the identification of CRISPR/Cas9-edited mutants (Le Trionnaire et al., 2019).

As CRISPR/Cas-mutational pipelines become more established, experiments will shift from the required technology development phase to addressing biological questions in the Hemiptera. This shift has begun for the brown planthopper, *N. lugens*. Pioneering advances in CRISPR/Cas9-editing of *N. lugens* began in 2018 and, by 2021, the analysis of CRISPR/Cas9-edited null mutants has provided critical and unequivocal new insights into roles of the pair of insulin receptors (*NlInR1* and *NlInR2*) (Zhao et al., 2019; Xue et al., 2021). As there is no ortholog of *InR2* in the model species *D. melanogaster*, the non-model insect *N. lugens* has already shed important light on *NlInR2* neofunctionalization (Xue et al., 2021).

Progress in developing gene-editing technologies in Hemiptera and the steps remaining are shown in **Figure 5**. The next advances will depend on the accelerated development of the core tools needed for robust genetic analysis in the Hemiptera. We need a battery of promoters with different specificities to drive target genes, as well as reporter genes suited for robust screens in embryos and adults of non-model hemipterans. We need transposable elements and their associated transposases that function efficiently in the Hemiptera to carry and integrate their genetic cargo in the target insect genomes; this will enable the identification of genomic safe harbors for insertion of genes and gene cassettes needed to explore the biology, ecology and control of the Hemiptera. We need to develop of gain-of-function protocols for gene integration *via* CRISPR/Cas9-mediated homology directed repair, as well by nonhomologous end joining using

CRISPaint protocols. This is likely to necessitate the development of hemipteran strains expressing Cas9 to enhance the frequency of these events. Finally, it is time to explore the advantages of other Cas endonucleases and the wide variety of CRISPR-mediated control strategies that can be deployed to interrogate gene regulatory programs in the Hemiptera.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.900785/full#supplementary-material

Supplementary Table S1 | Genome projects of Hemiptera.

Supplementary Table S2 | Cell culture lines of Hemiptera.

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REVIEW
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Genetic Approaches for Controlling CRISPR-based Autonomous Homing Gene Drives

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CRISPR-based autonomous homing gene drives are a potentially transformative technology with the power to reduce the prevalence of, or even eliminate, vector-borne diseases, agricultural pests, and invasive species. However, there are a number of regulatory, ethical, environmental, and sociopolitical concerns surrounding the potential use of gene drives, particularly regarding the possibility for any unintended outcomes that might result from such a powerful technology. Therefore, there is an imminent need for countermeasures or technologies capable of exerting precise spatiotemporal control of gene drives, if their transformative potential is ever to be fully realized. This review summarizes the current state of the art in the development of technologies to prevent the uncontrolled spread of CRISPR-based autonomous homing gene drives.

Keywords: CRISPR, Cas9, autonomous, homing, gene drive, controlling systems, braking systems

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1 INTRODUCTION

The ability to autonomously drive a trait of interest through a natural population in order to effect genetic control is a long sought-after goal. Early attempts at realizing this goal focused on the co-option of site-specific homing endonuclease genes [HEGs; (Burt, 2003, 2014; Windbichler et al., 2007, 2011; Chan et al., 2011)]. HEGs are naturally occurring selfish genetic elements that encode a nuclease, which recognizes and cleaves a 15–30 bp sequence that typically occurs only once in the genome. HEGs occurring in the middle of their own recognition site can then be used as a repair template and copied over into the cleaved site through the cell's DNA repair processes (Burt and Koufopanou, 2004). However, HEGs are constrained by highly specific protein-DNA interactions, which restricted design choices and limited their applicability to target new locations. The discovery of CRISPR-based genome editing opened the door to the development of more facile and powerful genome engineering tools by making nearly every nucleotide sequence in the genome accessible to editing (Doudna and Charpentier, 2014). As a result, since 2015, the development of CRISPR-based autonomous drives has progressed rapidly in both model and non-model organisms (Gantz and Bier, 2016; Hammond et al., 2016; Kyrou et al., 2018; Oberhofer et al., 2019; Simoni et al., 2020).

A major hurdle arising early in the development of CRISPR-based autonomous drives was the appearance of target alleles that are refractory to further Cas9 cleavage. These refractory target sites, termed "resistant" alleles, occurred as a consequence of either existing genetic variation, *de novo* mutation, or erroneous repair of the DNA double stranded breaks (DSBs) created by Cas9. In any case, the resistant allele prevents optimal drive transmission, and if selected for will ultimately remove the drive allele from the population. However, several studies have recently shown that this problem can be mitigated, or overcome completely, by directing the Cas9 nuclease to highly conserved loci, targeting the allele with multiple guide RNAs, and restricting the expression of Cas9

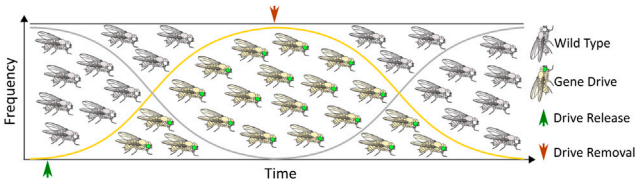

FIGURE 1 | The ideal gene drive: An autonomous homing gene drive designed to spread rapidly through a target population that is also amenable to scar-free excision leading to restoration of wild type alleles.

to early germline cells (Noble et al., 2017; Champer et al., 2018, 2020; Kyrou et al., 2018; Carballar-Lejarazú et al., 2020; Hammond et al., 2021).

Although some technical challenges remain, rapid advancements in the design and development of gene drives capable of mitigating the selection of genetic resistance alleles suggests that the development of technologies for limiting the spread of drives through a population will be necessary, if such tools are ever to be successfully tested and deployed in the field. The ideal technology would permit completely efficient genetic drive, but if necessary could be induced to initiate a process ultimately leading to the removal of the drive element, and complete restoration of the population to the original wild-type state (**Figure 1**). While no such technology currently exists, a number of systems for controlling CRISPR-based autonomous homing gene drives have been proposed, with many having already demonstrated proof-of-principle in model or non-model organisms. While progress in this area has not proceeded as rapidly as the development of the drives themselves, these studies provide valuable insights into the design and implementation of systems for managing the risks inherent to conducting genetic engineering on a massive scale. Thus, we summarize here the current theoretical and technological advancements made in controlling CRISPR-based autonomous homing drives (**Figure 2**; **Table 1**), assessing the strengths and weaknesses of each, and where applicable suggesting improvements that may be necessary for the approval and application of these innovative strategies. We have organized this information by organism, where appropriate. Although several designs for split/daisy drives have also been experimentally demonstrated as effective methods for controlling the spread of gene drives through a population, these are not included here as this review instead focuses on the development of technologies that could be incorporated into a single autonomous drive element.

1.1 Yeast

1.1.1 Synthetic Allele

Various systems for controlling CRISPR-based autonomous homing gene drives have been demonstrated in the unicellular eukaryotic model organism, *Saccharomyces cerevisiae*. One such system involves the introgression of a neutral synthetic DNA sequence into a natural population prior to the deployment of an autonomous drive. The drive is then programmed to target the introduced sequence, thereby restricting its spread only to the genetically modified organisms. This strategy establishes an effective barrier against unwanted genetic drive into non-target organisms or neighboring populations of the same species. In proof-of-principle studies, gene drive was observed in cells bearing the synthetic sequence at frequencies >99%, and not at all in yeast with wild-type sequences (DiCarlo et al., 2015). This strategy has some attractive features, and is relatively trivial to achieve in a laboratory setting. However, genetically modifying natural populations of multicellular organisms prior to drive release would be orders of magnitude more complex, with the introgressed sequences subject to Mendelian rates of inheritance, and very likely undesirable. Thus, this strategy is probably better suited to the confinement of drives in laboratory settings as an effective safeguard against escape. Naturally occurring sequence polymorphisms present in isolated populations might be amenable to such a strategy, so called precision drive; however, similar to HEG target sites these would likely be relatively rare and of limited utility (Esvelt et al., 2014).

1.1.2 Reversal Drive

Follow-up drives have been proposed to be used as "braking systems", halting and even reversing genome alterations affected by an earlier drive that has already spread through a population. One such system employs what has been termed an "overwriting drive", which carries a functional copy of the gene disrupted by the initial drive (a rescue gene), a source of Cas9, and a guide RNA that targets the initial drive. In yeast, the overwriting drive replaced the initial drive at an efficiency >99%, in subsequent generations restoring the function of the gene that was initially targeted by the earlier drive (Esvelt et al., 2014; DiCarlo et al., 2015). The overwriting process is confined to drive-bearing organisms, but only replaces one drive with another, leaving behind genetically modified transgenic organisms, which is likely to be undesirable considering the underlying motivation for removing the first drive may well apply to the second one as well.

1.1.3 Programmable Drive

Cas9-based autonomous drives invariably contain a nuclease, Cas9, and guide RNA (sgRNA). Thus, the expression, localization, or other properties of these components can be programmed to modulate the transmission rates of the drive element. Multiple independent mechanisms have been demonstrated to be capable of modulating drive transmission, ranging from complete efficiency to no activity. These mechanisms include: 1) titrating the level of Cas9 by controlling expression from the promoter 2) modifying the nuclear localization of Cas9 by including various combinations of nuclear export signals and nuclear localization signals 3) altering the efficiency of Cas9 targeting by varying the length or number of mismatches present in the sgRNA 4) generating tandem fusions with the *S. pyogenes* Cas9 and an enzymatically inoperative or dead dCas9 variant that serves as a competitor in binding an sgRNA (Roggenkamp et al., 2018; Goeckel et al., 2019). The platform developed in yeast provides a system for rapidly testing various tunable aspects of, or evaluating new methods for programming the performance of, gene drive constructs. The discoveries made in yeast, however, will need

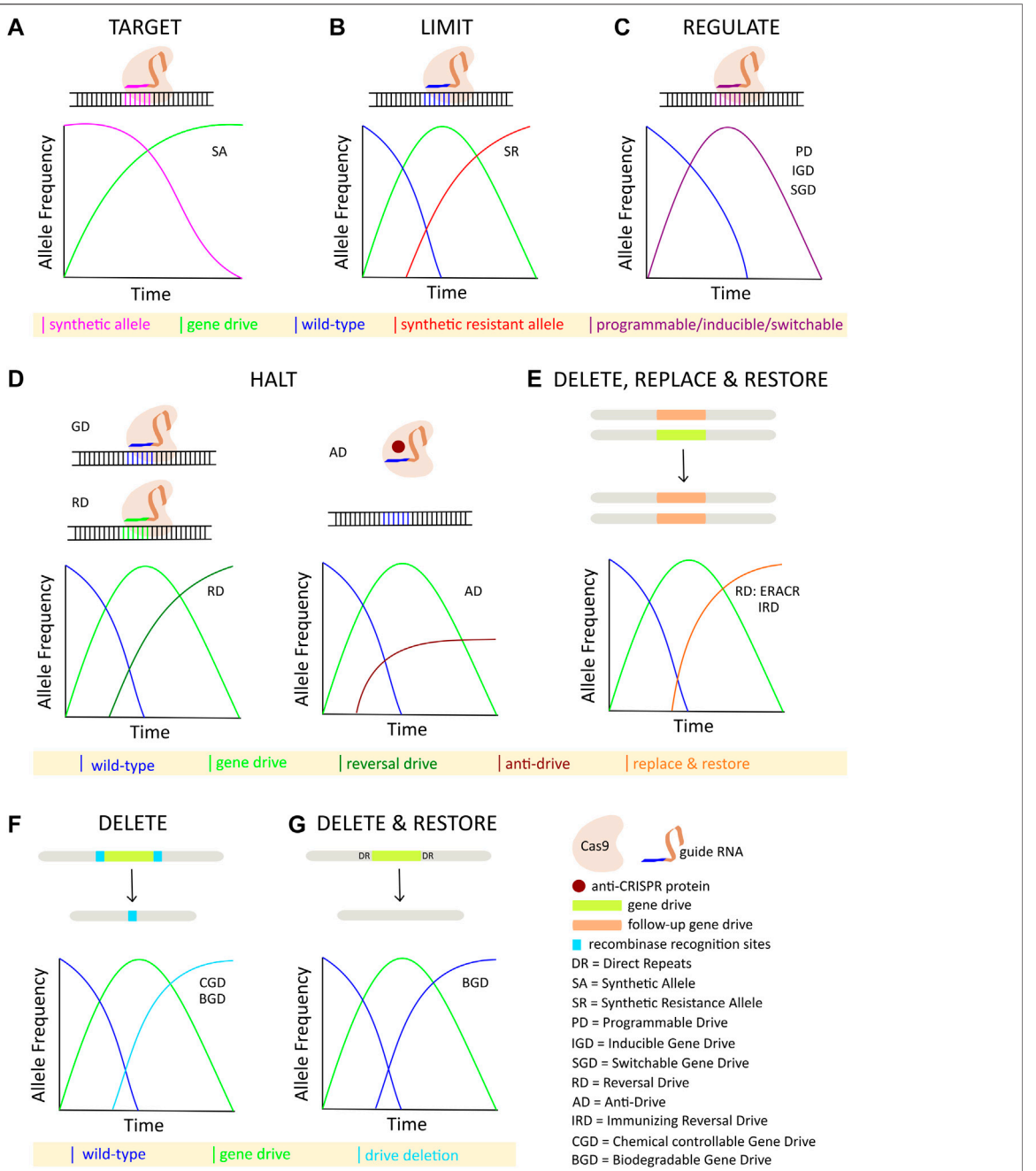

FIGURE 2 | Overview of strategies for controlling CRISPR-based autonomous homing gene drives: **(A)** Synthetic target sites are specifically cleaved and homed into by the autonomous drive element. **(B)** Inundation with synthetic resistant alleles limits the spread of an autonomous drive. **(C)** Titration and/or induction of various programmable, inducible, or switchable components of the autonomous drive regulate its spread. **(D)** Spread of autonomous drive is halted either by a second drive targeting the first drive, or by a protein-based anti-Cas9 interaction. **(E)** Autonomous drive is deleted and replaced with a second element carrying a rescue gene. **(F)** Autonomous drive is deleted by inducing recombination between transgenic target sites flanking the drive allele. **(G)** Autonomous drive is deleted by homology-based intramolecular recombination leading to restoration of the native wild-type alleles.

to be translated to sexually reproducing multicellular organisms. Further, as with most of the strategies summarized here, each of these mechanisms would need to achieve a "Goldilocks" zone of activity, where the gene drive is not hampered enough to prevent the achievement of population control or replacement over a specified period of time before ultimately being inactivated, which in practice might be difficult. Also similar with most of the other strategies, transgenic populations would be likely to persist for an extended period of time, unless the drive allele was associated with a significant fitness cost.

TABLE 1 | Studies on controlling CRISPR-based autonomous homing gene drives.

Drive control	Acronym	Control mechanism	Intended outcome	Species	References
Synthetic Allele	SA	Sequence polymorphism	Target specific populations of a species	Yeast	DiCarlo et al., (2015)
Reversal Drive	RD	Overwriting Drive	Halt or delete gene drives	Yeast	(Esvelt et al., 2014; DiCarlo et al., 2015)
		ERACRs, e-CHACRs		*Drosophila*	(Gantz and Bier, 2016; Xu et al., 2020)
		CATCHA		*Drosophila*	Wu et al., (2016)
		Cas9 deactivation		Theoretical	(Vella et al., 2017; Girardin et al., 2019; Rode et al., 2020)
Programmable Drive	PD	Cas9 and sgRNA programming	Titrate/regulate drive propagation	Yeast	(Roggenkamp et al., 2018; Goeckel et al., 2019)
Anti-Drive	AD	Anti-CRISPR proteins	Halt gene drive spread	Yeast	Basgall et al., (2018)
				Anopheles	Taxiarchi et al., (2021)
Chemical controllable Gene Drive	CGD	Small molecule-induced "off switch"	Excise gene drive without restoration of wild-type	*Drosophila*	Chae et al., (2020)
Inducible Gene Drive	IGD	Small molecule-induced "on switch"	Spatiotemporal regulation of gene drive activity	*Drosophila*	López Del Amo et al., (2020)
Switchable Gene Drive	SGD	Genetic code expansion	Spatiotemporal regulation of gene drive activity	Mouse	Suzuki et al., (2018)
Synthetic Resistance	SR	Synthetic resistance alleles	Drive extinction through introgressed resistance	Theoretical	(Burt, 2003; Vella et al., 2017; Rode et al., 2019)
Immunizing Reversal Drive	IRD	Recoded functional gene	Replace initial gene drive and wild type with a second drive carrying a functional recoded allele	Theoretical	(Esvelt et al., 2014; Vella et al., 2017; Rode et al., 2020)
Biodegradable Gene Drive	BGD	Self-elimination	Excise gene drive with or without restoration of wild-type	Theoretical	Zapletal et al., (2021)

1.1.4 Anti-Drive

Naturally occurring anti-CRISPR (Acr) inhibitor proteins have been identified in several bacteriophages (Dong et al., 2017; Rauch et al., 2017; Liu et al., 2019). Acr proteins are DNA mimics that directly interact with the Cas9 protein to competitively inhibit binding with the sgRNA-guide sequence and subsequent Cas9-mediated cleavage. In a haploid yeast model, gene drive homing was almost completely inhibited by the anti-CRISPR peptides, AcrIIA2 and AcrIIA4 (Basgall et al., 2018). Integration of these peptides into the gene drive construct under inducible promoters permitted drive inhibition to be titrated to levels that were lower than the nearly complete abrogation observed previously. However, the inducible promoter used did exhibit leaky expression of the AcrIIA2/AcrIIA4 transcripts, which has been commonly observed in most if not all inducible systems tested. Further, it is not clear how such a system might be implemented outside of a highly controlled laboratory environment. An earlier study demonstrated that the activity of these proteins is sensitive to the temporal and spatial presence of the inhibitor (Shin et al., 2017).

Intriguingly, mutational scanning revealed several amino acid positions that resulted in a partial loss of inhibitory activity, suggesting another way that drive inhibition might be titrated (Basgall et al., 2018). Such strategies would face the same challenges as other tunable systems, which are described above. Anti-drive systems indeed have great potential in halting the unwarranted spread of Cas9-based autonomous homing gene drives, but will need to be evaluated further in sexually reproducing diploid organisms.

1.2 *Drosophila*

1.2.1 Reversal Drive

Several reversal drives have been developed in the model organism, *Drosophila melanogaster*. These drives have also been referred to as braking systems. Braking systems are based on various designs of transgenic genetic elements that carry one or more sgRNAs, but are devoid of any source of Cas9. These elements are typically designed to target the Cas9 sequence within an autonomous drive that has already spread through a population. The sgRNA or sgRNAs derived from the braking element associate with the Cas9 protein produced from the drive allele, either mutating the Cas9 sequence, or replacing the drive allele with that of the reversal element (Gantz and Bier, 2016; Wu et al., 2016; Xu et al., 2020).

For example, in the eCHACR (erasing construct hitchhiking on the autocatalytic chain reaction) iteration, a neutralizing element is inserted into the genome at a location independent of the drive allele, but is capable of self-copying and inactivating the Cas9 sequence of the gene drive. The eCHACR construct functions by exploiting the tendency of the Cas9 nuclease to generate alleles resistant to further cutting, resulting from erroneous repair of the double stranded break through non-homologous end joining (NHEJ) (Xu et al., 2020). The eCHACR

concept was demonstrated to be capable of neutralizing a gene drive in multi-generation cage trials. However, eCHACRs do not delete the original gene drive element. Additionally, the neutralizing element itself introduces an additional transgene into the population. In a similar approach, a CATCHA (Cas9-triggered chain ablation) transgene is inserted directly into the Cas9 sequence of the drive element, inactivating the nuclease (Wu et al., 2016). In the eCHACR and CATCHA technologies, the genetic element may encode one or more sgRNAs. These sgRNAs may be directed at the insertion site to facilitate gene conversion and self-copying, or at the Cas9 sequence of the gene drive allele in order to inactivate it. In another design, an ERACR (element reversing the autocatalytic chain reaction) self-copying element is inserted directly into a locus in the drive allele, thereby deleting and replacing it (Xu et al., 2020). ERACRs encode two different sgRNAs, but they are directed at target sites flanking the drive element to facilitate its exchange with the ERACR transgene. ERACR elements may be engineered to carry an in-frame re-coded portion of the disrupted target gene, restoring its function. In multi-generation population studies, ERACRs were found to efficiently replace a gene drive. However, in some cases the ERACR element produced unexpected recombination events, damaging the target chromosome, and causing negative fitness effects (Xu et al., 2020). In other cases, the gene drive remained, again through the generation of alleles resistant to further cutting by the Cas9 nuclease. In each of these designs, the neutralizing elements encode sgRNAs, but rely on the Cas9 from the gene drive to act *in trans*, encoding none of their own.

1.2.2 Chemical controllable Gene Drive

The spread of an autonomous homing gene drive can be controlled by incorporating a chemically responsive "off switch". Such a chemically controllable gene drive (CGD) cassette has been attempted with the following components: 1) an autonomous homing gene drive element 2) a site-specific recombinase (Rippase) driven by a modified GAL4/UAS system, activated by RU486 (GeneSwitch). The CGD cassette is flanked by recognition sites for Rippase, which aid in the removal of the entire cassette via recombination (Chae et al., 2020). However, in population studies the CGD system was only minimally effective, with the gene drive being eliminated relatively inefficiently. The authors of the study identified several limitations of their experimental design, and acknowledged the need for higher statistical power and optimization of the RU486 effect. In addition, some components of the system are unlikely to be feasible outside of a laboratory environment.

1.2.3 Inducible Gene Drive

An alternative to an "off switch" is an "on switch," where Cas9-based gene drive activity can be induced by a small molecule. One such inducible gene drive (IGD) system was described in a non-autonomous split gene drive system, where the Cas9 and sgRNAs are encoded in separate loci on the same or different chromosomes (CopyCat drive system). In this system, the Cas9 protein is fused to an unstable protein domain (dihydrofolate reductase (DHFR) from *Escherichia coli*) that is targeted for proteasomal degradation upon expression (DD-*Sp*Cas9). Addition of a small molecule ligand Trimethoprim (TMP) stabilizes DD-*Sp*Cas9, enabling CopyCat drive activity. In a proof-of-principle study, addition of TMP stabilized the Cas9 fusion protein and facilitated a dose-dependent super-Mendelian inheritance of both the DD-*Sp*Cas9 and sgRNA constructs (López Del Amo et al., 2020). Differential homing efficiencies were observed for sgRNA constructs targeting the *white* and *ebony* genes, with the *white* CopyCat drive system producing relatively higher homing rates with increasing doses of TMP. The inducibility of IGDs facilitates spatiotemporal control, which is a highly desirable quality in the development of gene drive technologies. Similar to the CGD "off switch", and other inducible systems, IGDs also suffer from leakiness, which has proven to be a universal technical challenge in developing any ligand-based system of control. If the leakiness that seems to be an inherent feature of nearly all inducible systems evaluated so far can be addressed, IGDs should be thoroughly tested in autonomous homing gene drive systems, as split configuration drives, such as the CopyCat system, cannot function as low-threshold gene drives when released into natural populations.

1.3 Mosquito

1.3.1 Anti-Drive

In the human malaria vector, *Anopheles gambiae*, highly efficient Cas9-based autonomous suppression drives have been developed and evaluated (Kyrou et al., 2018; Hammond et al., 2021). Suppression drives typically target genes that are functionally constrained and cause recessive female sterility, reducing fecundity and resulting in population suppression. As a countermeasure to these highly efficient drives, an anti-drive system was developed consisting of the anti-CRISPR peptide, AcrIIA4. The AcrIIA4-based anti-drive system inhibited homing of two different Cas9-based suppression gene drives targeting either the fertility gene, AGAP007280 (*Drosophila* orthologue of *nudel*) or the sex determination gene, *doublesex*. In cage trials, introgression of anti-drive males prevented population suppression by the drive targeting *doublesex* (Taxiarchi et al., 2021).

Unlike reversal drives that rely on nuclease-based cutting of DNA, anti-drive systems have not been associated with unintended genome alterations at or near the drive target site, as they are premised on protein-protein interactions. Anti-drive systems that are not incorporated into the gene drive construct will be subject to normal rates of Mendelian inheritance, as they do not include any homing mechanism. Thus, the persistence of these constructs in a population is determined by the relative fitness associated with the anti-drive element. Anti-drive alleles associated with relatively low fitness costs may persist in populations, establishing a general barrier to any new introductions of CRISPR-based gene drives, which may or may not be a desirable feature (Taxiarchi et al., 2021). Incorporation of an inducible anti-drive system into an autonomous homing gene drive construct might permit spatiotemporal titration of drive transmission, but would also need to overcome all of the challenges associated with other inducible systems (elaborated on above).

1.4 Mouse

1.4.1 Switchable Gene Drive

Inducible systems that employ small molecules, such as RU486 and Trimethoprim, often exhibit leakiness, which presents problems for applications in regulating gene drives (Chae et al., 2020; López Del Amo et al., 2020). Further, while many of these systems are commonly used in a laboratory setting, environmental use would be both unsafe and impractical. For example, RU486 or mifepristone, is a component of a drug combination often used to induce abortions. Genetic code expansion, which is widely used in synthetic biology, has been proposed as a method for generating Cas9 variants that could function as part of an alternative system for achieving non-leaky control over the expression of the nuclease (Suzuki et al., 2018; de la Torre and Chin, 2021). Such Cas9 variants contain one or more modified codons that incorporate non-canonical amino acids (ncAAs) only in the presence of the corresponding orthogonal aminoacyl-tRNA synthetase/tRNA (aaRS/tRNA) pairs. For example, lysine codons within the Cas9 sequence were replaced with an amber stop codon (UAG) to produce a truncated non-functional protein. However, in the presence of an orthogonal aaRS/tRNA pair that recognizes and incorporates an ncAA, H-Lys (Boc)-OH (BOC), at the codon, a full-length functional Cas9 protein was produced. Expression of the BOC-inducible Cas9 ($Cas9^{BOC}$) was not leaky, and capable of editing both a reporter sequence (*eGFP*) and two different endogenous genes (*Sry* and *Tyr*) in mouse embryos. However, editing of the reporter gene was somewhat limited with either Cas9 or the $Cas9^{BOC}$, presumably due to position effects (Suzuki et al., 2018). Nevertheless, the study demonstrates that ncAA-mediated control of Cas9 expression, through expansion of the genetic code, can be a valuable tool in developing stringent control over Cas9-based gene drives. However, relatively extensive genome engineering will be required to generate organisms with the essential components.

1.5 Theoretical Models

1.5.1 Synthetic Resistance

Resistant alleles have the potential to be strongly selected for when insertion of an autonomous homing drive is associated with a highly deleterious phenotype. Thus, the introduction of organisms with target genes possessing fully functional resistant alleles could act as a braking system that is capable of extinguishing a gene drive. This approach is predicted to be more effective against drives that are associated with high fitness costs and large genetic loads (i.e., suppression drives), but less effective against drives that are associated with low fitness costs (i.e., modification/replacement drives). This is because natural selection will strongly favor functional, but drive-resistant, alleles with little to no fitness cost over drive alleles with higher fitness costs (Burt, 2003; Vella et al., 2017; Rode et al., 2019). Deterministic models indicate that to effectively remediate a drive-containing population the introduction of synthetic resistance alleles would need to achieve high thresholds in order to counter ongoing conversion of wild-type alleles to gene drive alleles (Vella et al., 2017).

1.5.2 Reversal Drive

As reversal drives are designed to target Cas9 sequences within autonomous drives, they are not predicted to affect wild-type alleles. Deterministic models indicate that at high release frequencies reversal drives will rapidly establish an equilibrium with wild-type alleles. However, at low release frequencies an initial increase in drive alleles will subsequently be followed with an increase in reversal drive alleles, ultimately reducing the frequency of drive alleles in the population (Vella et al., 2017). Other deterministic models that view drive/brake/wild type alleles in a rock/paper/scissors scenario find that coextinction of the drive/brake alleles is only possible when the fitness costs associated with these alleles are small enough (Girardin et al., 2019). Stochastic models of autonomous suppression drives that consider variable population size, but not the evolution of resistant alleles, indicate that braking systems might only be effective with threshold-dependent drives (Rode et al., 2020).

1.5.3 Immunizing Reversal Drive

Immunizing reversal drives are designed to recall unwanted drives while restoring gene function. Immunizing reversal drives typically carry multiple guide RNAs, their own source of Cas9, and a re-coded copy of the wild-type gene. Immunizing reversal drives target both the unwanted initial drive and wild-type alleles. The re-coded functional gene copy not only protects the IRD against being targeted by the initial drive (immunizing), but also restores normal gene function (reversal) (Esvelt et al., 2014). Deterministic models indicate that IRDs rapidly reach fixation, irrespective of release size. This is because IRDs replace both the unwanted initial drive and wild-type alleles, and therefore do not coexist with the other alleles in a polymorphic equilibrium (Vella et al., 2017). Stochastic models also recommend IRDs as the preferred braking system, as they restore fitness and are likely to spread through a population more quickly (Rode et al., 2020). Therefore, IRDs might represent the fastest way to counter and replace unwanted drives. However, deterministic modeling of suppression drives that naturally generate resistant alleles indicate that IRD alleles will be associated with relatively lower fitness than these alleles, and would eventually fall out of the population (Vella et al., 2017).

1.5.4 Biodegradable Gene Drive

A biodegradable gene drive (BGD) can be described as an autonomous homing transgene that can be pre-programmed to self-eliminate (*via* intramolecular recombination) after achieving its intended goals. Biodegradable gene drives are composed of an autonomous homing element (e.g., Cas9 nuclease with sgRNA) and a self-eliminating element (a second nuclease or pair of nucleases with one or more target site(s) in the transgene). The nuclease in the self-eliminating element can be a recombinase, integration-deficient transposase, or endonuclease. While recombinase-mediated excision of a BGD would leave behind one of its two target sites, transposon or endonuclease-mediated BGD

excisions would result in the restoration of wild-type alleles. Deterministic models of BGDs, simulating suppression drives, indicate that autonomous drive transmission would be rapidly reversed, even with a relatively modest rate of self-elimination (<10%) (Zapletal et al., 2021). As BGDs result in the restoration of wild-type alleles and the removal of transgenes, they may represent a crucial development in addressing legitimate ethical, regulatory, and environmental concerns associated with gene drive technologies. However, at the moment BGDs remain purely theoretical and a number of technical challenges will undoubtedly need to be overcome in order to develop working technologies that can subsequently be investigated in laboratory and field settings. For example, inclusion of self-eliminating activity at the point of drive release may face many of the same challenges as other strategies (elaborated on above). Similarly, while an inducible system that activates the self-eliminating mechanism might be preferable, a system enabling tight spatiotemporal control over autonomous drive transmission in either a laboratory or field setting remains to be identified, tested and optimized.

2 CONCLUSIONS AND FUTURE DIRECTIONS

CRISPR-based autonomous homing gene drives are an innovative and potentially transformative technology. An important aspect of responsible conduct of research into such a powerful technology is the development of countermeasures that can if required halt or reverse any unwanted or undesired outcomes. These countermeasures should be sufficiently mature before any gene drive technologies are approved for biocontrol, not only in the event of an emergency, but also to potentially recall a drive that has achieved its intended goals.

Nearly all of the controlling systems developed so far are designed to stop and/or reverse the drive, but rarely address the environmental persistence of transgenes. Both empirical studies and modeling of braking systems have found that transgene persistence is dependent on the fitness costs associated with both the drives being targeted and the braking systems themselves. With regard to reversal drives, IRDs are predicted to be the most efficient braking systems; however, relatively high fitness costs may cause them to fall out of a population (Vella et al., 2017). Further, immunizing reversal drives have not yet been extensively tested in laboratory settings, so modeling results still need to be confirmed with *in vivo* studies. Several other types of reversal drives have been developed and tested, both empirically and theoretically. While these might be useful in emergencies, to halt the spread of an unwanted drive, evaluation of these systems is far from complete. Large-scale laboratory experiments designed to evaluate the ability of braking systems to stop or replace a gene drive have revealed unintended genetic outcomes that included the introduction of substantial fitness costs and the persistence of transgenes retaining full or partial drive potential. In this regard, anti-drive systems that halt drive activity through protein-protein interactions might be preferable, as these systems have not been associated with unintended genome alterations, but similarly require further testing and modeling under various scenarios. Biodegradable gene drives incorporating self-elimination mechanisms also might be a viable alternative, with the potential to directly address transgene persistence through mechanisms capable of replacing drive alleles with wild-type alleles. However, at the moment, BGDs are largely theoretical, requiring extensive research and development.

A number of gene drive strategies currently under development are based on the premise of population suppression. In these scenarios, a drive allele would introduce a fitness load or gender bias into a target pest species. However, the enormous selection pressures that will be brought to bear on these species from these gene drive-based approaches would be likely to strongly select for any resistant genotypes that might be generated through genetic mechanisms, which are difficult to predict or control. Thus, the eventual outcomes of such approaches may be similar to that of the DDT-based Global Malaria Eradication Program, which was initially very successful, but ultimately resulted in the development of widespread DDT resistance in mosquito populations. Spatially and temporally limiting gene drives might prevent the evolution of resistance to gene drive strategies by reducing these selection pressures. While such an approach would likely require multiple releases, this might be preferable to the generation of resistance. The evolution of resistance to gene drive activity might not be limited to the presence or generation of fitness costs or unexpected genomic events. Naturally occurring selfish genetic elements (i.e., transposons) are a constant source of genetic conflict and subject to control by genomic defenses (reviewed in Werren et al., 1988; Kazazian, 2004; McLaughlin and Malik, 2017). Extensive and widespread use of synthetic Cas9-based gene drives may ultimately result in similar genetic conflicts, leading to targeting by evolutionarily conserved small RNA pathways, such as those generating PIWI interacting RNAs (piRNAs) and short interfering small RNAs (siRNAs), which might be difficult to circumvent. Finally, it seems unlikely that any current technology can adequately address all of the concerns associated with the use of gene drives. Therefore, it is important that many different control strategies continue to be investigated and considered.

AUTHOR CONTRIBUTIONS

PC wrote the initial draft and KM edited the draft. All authors contributed to manuscript revision, read, and approved the submitted version.

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Manipulating Insect Sex Determination Pathways for Genetic Pest Management: Opportunities and Challenges

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Sex determination pathways in insects are generally characterised by an upstream primary signal, which is highly variable across species, and that regulates the splicing of a suite of downstream but highly-conserved genes (*transformer*, *doublesex* and *fruitless*). In turn, these downstream genes then regulate the expression of sex-specific characteristics in males and females. Identification of sex determination pathways has and continues to be, a critical component of insect population suppression technologies. For example, "first-generation" transgenic technologies such as fsRIDL (Female-Specific Release of Insects carrying Dominant Lethals) enabled efficient selective removal of females from a target population as a significant improvement on the sterile insect technique (SIT). Second-generation technologies such as CRISPR/Cas9 homing gene drives and precision-guided SIT (pgSIT) have used gene editing technologies to manipulate sex determination genes *in vivo*. The development of future, third-generation control technologies, such as Y-linked drives, (female to male) sex-reversal, or X-shredding, will require additional knowledge of aspects of sexual development, including a deeper understanding of the nature of primary signals and dosage compensation. This review shows how knowledge of sex determination in target pest species is fundamental to all phases of the development of control technologies.

Keywords: gene drive, sex conversion, release of insects carrying a dominant lethal, sterile insect technique (SIT), doublesex (dsx), *tra*, dosage compensation, sex determination'

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INTRODUCTION

Insect pests cause enormous damage to human health (through the transmission of diseases such as dengue fever and malaria) and agriculture (through damage to crops or livestock). Existing control methods include pesticides, biological control, and integrated pest and habitat management. However, while many of these approaches have been highly successful, they also have limitations. For example, the use of pesticides can select strongly for resistance, damage non-pest populations (Hawkins et al., 2018) and the environment. The success of biological control and integrated management programmes may also depend upon whether efficient natural enemies are available and the specific ecological setting. As a result, existing control strategies, particularly chemical control, are likely to become increasingly restricted while simultaneously becoming less effective. Global climate change is also predicted to increase the

range and the number of insect pests (Deutsch et al., 2018; Gomez-Zavaglia et al., 2020; Sultana et al., 2020). Therefore, it is clear that there are significant challenges for the future in controlling insect pests, to safeguard against disease and maintain global food security.

In light of these concerns, there has been considerable investment in new and alternative technologies, such as genetics-based approaches to pest control, to protect health and food security (Alphey, 2014; Burt and Crisanti, 2018; Raban et al., 2020). Genetic Pest Management (GPM) aims to harness the natural mating system of the pest species to introduce into the target population traits that will reduce fitness and ultimately lead to a reduction of numbers or elimination. The most widely used GPM systems for suppression to date have been variants of the sterile insect technique (SIT) (Klassen, 2005), including the Wolbachia incompatible insect technique (IIT) (Atyame et al., 2011; De Barro et al., 2011; Zheng et al., 2019) and genetic engineering (Phuc et al., 2007). GPM systems that are transmitted or inherited through one sex and sterilise, kill or change the sex of the other offer the most significant potential for control (Bax and Thresher, 2009). As females are predominantly the agents of damage (*via* biting or ovipositing), and generally determine the effective population size, most approaches have focused on releasing benign males that produce either male progeny or none at all (Rendón et al., 2004). Elimination of females ensures long-term suppression and immediately reduces the associated damage caused by biting or egg-laying.

GPM technologies for insect population suppression currently under development seek to improve on older systems by spreading female-targeting genetic loads through a population or converting female progeny into functional males. These newer technologies also make wide use of contemporary molecular biology tools—particularly those involved in gene editing such as CRISPR/Cas9. However, what is common to all is that they exploit knowledge of the sex determination pathways of the target species, thus exemplifying the importance of incorporating fundamental biological principles to underpin applied science in GPM (Leftwich et al., 2016; Leftwich et al., 2021).

Here we first introduce the fundamentals of insect sex determination systems, focusing on species of interest to GPM. We detail which components are conserved and which show more rapid evolution, what types of primary signals have evolved and in which "direction" they push downstream cascades (e.g., towards maleness or femaleness). We then provide a framework for understanding how sex determination systems have been used to develop insect population suppression tools. We describe three "generations" of genetic engineering technologies with related components or goals. First-generation systems are genetically engineered analogues of the classical Sterile Insect Technique. Second-generation systems are under development and are made possible by the development of the CRISPR/Cas9 platform. Finally, we discuss the challenges inherent in developing "third-generation" control technologies that seek to achieve the goal of sex-conversion by manipulating master regulators of sex determination.

INSECT SEX DETERMINATION SYSTEMS

Sex determination systems can be described as a cascade in the form of a pyramid. There is an initial primary signal ("master regulator"—top of the pyramid) that initiates a limited series of intermediary elements (middle) that then result in diverse downstream sexual differentiation and development (base of the pyramid). In insects, the genes at the base of the pyramid tend to be highly conserved, while the elements at the top show marked diversity, both in identity and function (Adolfi et al., 2021; Hopkins and Kopp, 2021). The hypothesis is that these basal genes—which generally consist of transcription factors—represent ancient mechanisms of sex determination [e.g., *doublesex* is shared with some non-dipteran arthropods (Price et al., 2015; Wexler et al., 2019)]. At the same time, the primary signal can evolve rapidly, even within a taxonomic order. The differences in conservation between the basal and intermediate elements of the sex determination pathway are shown in **Figure 1**.

Amongst the downstream basal elements, the most well conserved is *doublesex (dsx)* (Saccone et al., 2002; Price et al., 2015; Verhulst and van de Zande, 2015; Wexler et al., 2019). Ubiquitous amongst insects, *dsx* is the "central nexus" between sex determination and sexual differentiation cascades (Verhulst and van de Zande, 2015). It functions as a transcription factor activating or repressing thousands of downstream genes which cause female or male somatic differentiation. Its role in this regulation (male or female biasing) is determined by whether it exists in a male or female "form" as a protein. In most cases, this is determined by sex-specific alternative splicing of the initial *dsx* pre-mRNA—itself determined by intermediary regulators between the primary signal and *dsx*. "Male" *dsx* typically represents the constitutive splicing isoform; while female-specific *dsx* isoforms require the splice enhancing factor transformer (*tra*) (**Figure 2**). However, even within this most conserved member of the insect sex determination cascade, some variation does exist. For example, there are significant differences in the number and "style" of exon skipping between different insect species (Verhulst and van de Zande, 2015). For example, in lepidopterans, the constitutive dsx isoform is female with male determining factors required to shift splicing towards a male form (Lee et al., 2015; Xu et al., 2017; Visser et al., 2021). Further, in at least two species of termites, *dsx* has evolved towards male-only expression rather than sex-alternate splicing (Wexler et al., 2019; Miyazaki et al., 2021).

An intermediary element that exists directly above *dsx* is *transformer* (*tra*). Although not as highly conserved as *dsx* (Verhulst et al., 2010b), *tra* homologues have been identified in a variety of insect orders, e.g., Coleopterans [*Tribolium castaneum* (Shukla and Palli, 2012)], Hymenopterans [*Apis mellifera* (Gempe et al., 2009), *Nasonia vitripennis* (Verhulst et al., 2010a)] and dipterans [*Drosophila melanogaster* (Sosnowski et al., 1989; Inoue et al., 1990), *Musca domestica* (Inoue and Hiroyoshi, 1986) and a number of Tephritid fruitflies (Pane et al., 2002; Lagos et al., 2007; Ruiz et al., 2007)]. In these groups, sex-specific splicing of *tra* leads to a "functional" female-specific or "non-functional" male-specific protein. Interestingly,

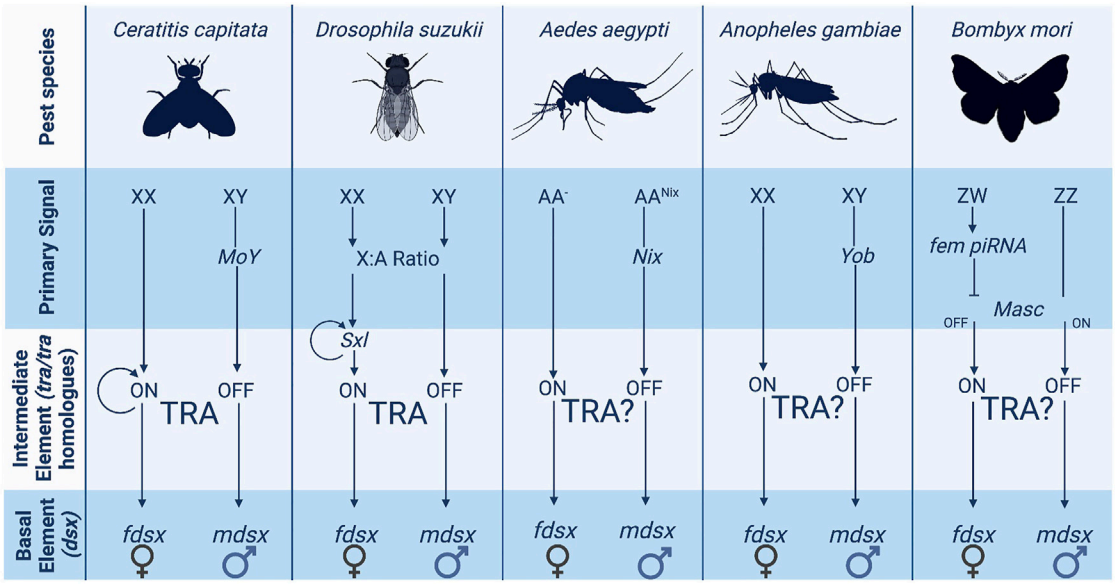

FIGURE 1 | The variety of sex determination systems employed by pest insects. The more upstream elements of the sex determination pathway such as the primary signal vary widely across pest insect species. Several species have XX/XY chromosome structures although they do not utilise them in the same way. Ceratitis and Aedes employ masculinizing elements carried on the Y chromosome whereas Drosophila uses X counting to determine sex.

tra has often been found to self-regulate its splicing (except in *D. melanogaster*) acting as a positive self-regulatory element to increase its own expression (Pane et al., 2002; Gempe et al., 2009; Salvemini et al., 2009; Verhulst et al., 2010a; Hediger et al., 2010). Functional *tra* acts as a splicing enhancer, binding *dsx* pre-mRNA and promoting the inclusion of female-specific exons in the final transcript.

Although *tra* is an essential gene in the sex determination pathway of many dipterans and hymenopterans; in other species, there may be, as yet, no identified homologue, as is the case for *Aedes aegypti* (Nene et al., 2007). In these cases, there may be functional quasi-equivalents for *transformer*. For example, in silkmoth (*Bombyx mori*) males, P-element somatic inhibitor and IGF-II mRNA binding proteins interact to form a complex which binds *dsx* pre-mRNA. This complex inhibits internal splice site junctions, excluding female-specific exons to produce "male" form dsx mRNA (Suzuki et al., 2010). This example illustrates that while there may be orthogonal splicing factor/s, analogous to *tra*, the role the new factor/s plays may be very different (promoting male-form, rather than female-form *dsx* alternate splicing). The *transformer-2* gene (*tra2*) is also involved in the sex determination pathway of many insects. It is often an additional factor that forms an essential part of the splicing enhancer complex, which helps sustain and regulate the splicing of *tra* (Salvemini et al., 2009). It is, however, not a homologue of *transformer* itself. *Tra-2*, unlike *tra,* has also been shown to have both expression and function in males (Salvemini et al., 2009).

Above *tra* (or other intermediary elements) in the sex determination pathway lies the primary signal or master regulator underpinning the sexual determination cascade. The identity and function of these master regulators vary enormously between species even within the same order due to a high turnover rate of the primary signaler at this level (Gempe and Beye, 2011). For example, in four dipteran species, the mosquitos *Anopheles gambiae*, *Ae. aegypti,* the Mediterranean fruitfly *Ceratitis capitata*, and the house fly *M. domestica*, the master regulators of sex determination are evolutionarily unrelated [*Yob, Nix, MoY*, and *Mdmd* respectively (Hall et al., 2015; Krzywinska et al., 2016; Sharma et al., 2017; Meccariello et al., 2019)]. While the exact mechanisms by which these primary signals act remains largely unknown, the mosquito species assessed so far (including those listed above) do not appear to possess a *tra* homologue (Nene et al., 2007). In contrast, the sex-specific splicing of *tra* is integral to the sex determination cascade in *C. capitata and M. domestica*, strongly suggesting divergent functions in regulating intermediary elements between the top and bottom levels of the pyramid in mosquitos and other diptera (Saccone et al., 2011). In *D. melanogaster*, sex is determined by an X-chromosome counting mechanism. The expression ratios of specific X-linked (*sis-a, sis-b, sis-c* and *run*) and autosomal genes determine the correct expression of an autoregulatory-splicing female-determining gene (*sex-lethal*) (Cline, 1993; Kaiser and Bachtrog, 2010). A 1:1 X: A ratio (implying two X chromosomes) leads to functional sex-lethal expression and the female sex determination cascade (Baker and Ridge, 1980; Sánchez and Nöthiger, 1982; Parkhurst et al., 1990). Although *tra* plays a crucial intermediary role in *D. melanogaster* (as in *C. capitata*), the sex-determining role of *sex-lethal* appears to be Drosophilid-specific (Meise et al., 1998). In Lepidoptera, primary signals can vary, as both Z-chromosome counting and dominant primary signals exist in different species (Traut et al., 2007). In *B. mori*, a

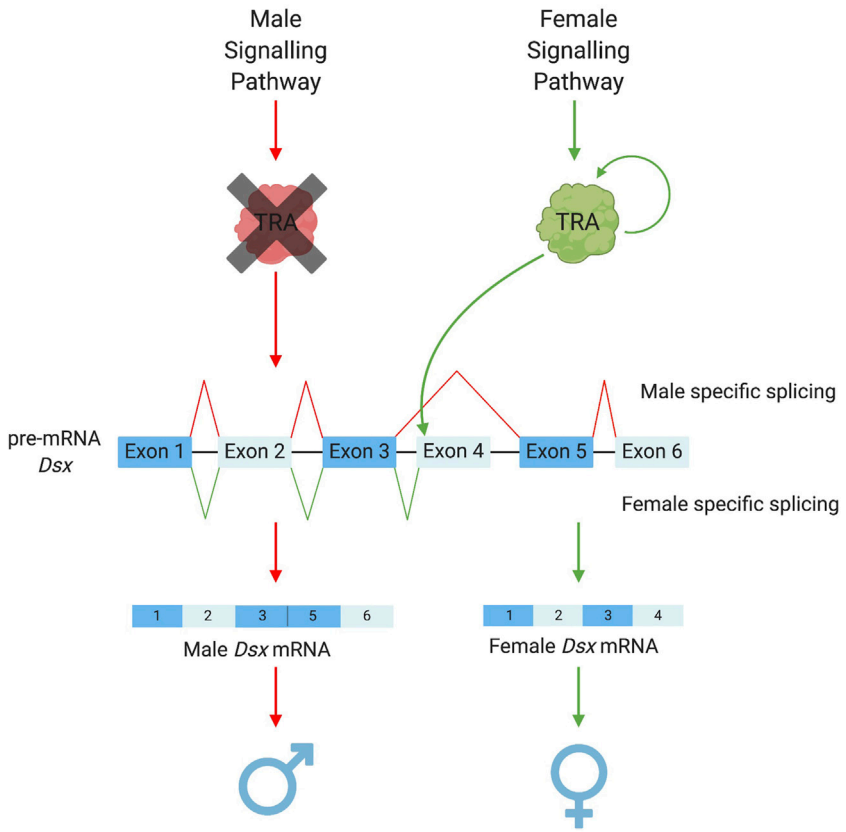

FIGURE 2 | Sex-specific splicing patterns of doublesex. The conserved elements of the sex determination pathway that result in the alternative splicing of the pre-mRNA dsx. Depending on the upstream signalling pathways TRA is either present as a functional protein (in female development) or a non-functional protein (in male development). TRA acts as a splicing enhancer to promote the recruitment of the splicing machinery to the weak splice acceptor prior to exon 4 of the dsx pre-mRNA (Shukla and Nagaraju, 2010). This allows for the retention of exon 4 in the mRNA resulting in the female version of dsx. Doublesex exon numbers vary among species with the use of splicing to retain or remove sex-specific exons remaining constant, Ceratitis capitata pathway illustrated in this figure.

dominant female-linked (*feminizer*) piRNA system encoded on the female-specific W chromosome silences Z-linked genes that would otherwise initial male sex-determination (Hasselmann et al., 2008; Katsuma et al., 2018); this then directs *dsx* splicing. In Hymenoptera, sexual fate is effectively regulated by the presence or absence of a paternal genome. E.g., in the honeybee, *A. mellifera*, it is determined by heterozygosity at a single locus the complementary sex determiner (csd) gene (Gempe et al., 2009), single sex alleles within an organism result in male development (homozygous/hemizygous) and mismatched sex alleles develop into females (heterozygous). While in the haplodiploid wasp *N. vitripennis* the sex determination gene *wasp overruler of masculinization* (*wom*) is only transcribed from the paternally provided genome (Zou et al., 2020). In both systems, *tra* and *dsx* are still employed as intermediate and basal elements (Cho et al., 2007; Zou et al., 2020).

The genomic location of the master regulator and, specifically, whether it exists on a heteromorphic sex chromosome is a further important aspect of insect sex determination systems in this review. In many cases, the evolution of distinct sex-chromosomes necessitates a mechanism for equalising the expression of shared, sex-linked genes between sexes (dosage compensation). In *D. melanogaster*, the absence of *sex-lethal* in males initiates hypertranscription from the single X-chromosome to make up for two X's in females. Inactivation of *sex-lethal* in females leads to deadly X-chromosome hypertranscription due to carrying two X chromosomes. This "coupled" sex determination and dosage compensation has implications for manipulating these systems for genetic pest control. For example, it is a significant challenge to aim to alter sexual fate without simultaneously "programming" the dosage compensation pathway. As a result, in many cases where the master regulator has been identified and ectopically expressed in females (e.g., *Yob* in *An. gambiae; Guy1 in Anopheles stephensi*), the result is the death of the XX individuals rather than their conversion to fertile males (Criscione et al., 2016; Krzywinska and Krzywinski, 2018; Qi et al., 2019). This represents a high hurdle if the most potent application of manipulating sex determination for GPM suppression systems is to convert a population to a single-sex rather than selectively kill off females.

Fortunately, neither fully differentiated sex chromosomes, nor coupled-dosage compensation pathways where heterogametic sex chromosomes exist, appear universal amongst insects that are of concern to human health or agriculture. For example, *Aedes albopictus* (and other culicine mosquitos) do not possess heterogametic sex chromosomes but rather a small "Male-determining" locus on chromosome 1 (Hall et al., 2014; Gomulski et al., 2018). Transgenic expression of the male-determining gene from within this locus (*Nix*) in transgenic *Ae. albopictus* was sufficient to convert females into functioning males (Lutrat et al., 2022). Similarly, in *C. capitata*, transient ectopic expression of *MoY* is enough to convert karyotypic females to functional males, suggesting either a lack of dosage compensation or an "un-coupled" version in this species (Meccariello et al., 2019).

Understanding the nature of the sex determination pathway that has evolved in a pest of concern, and its possible interaction with dosage compensation, provides some potential routes for manipulating a species for genetic pest management. If the goal is female to male sex *conversion*, then the upper levels of the pyramid will likely need to be manipulated to ensure complete sex conversion and the viability/fertility of converted individuals. However, this goal may be difficult or practically impossible for species with lethal dosage compensation. If the goal is simply to kill one of the sexes, then lower, more conserved levels of the pyramid can be targeted, and this may also be beneficial in transferring efficient designs between related pests. Further exploration of the fundamental basis of sex determination mechanisms is, therefore, essential.

FIRST-GENERATION TECHNOLOGIES: AN IMPROVEMENT ON THE PAST

First-generation transgenic GPM systems are genetically engineered analogues of the classical sterile insect technique (SIT). In the SIT, the mass release of irradiated (sterilised) males results in a lower proportion of fertilised females in the field due to mating with the sterile males instead of the fertile, wildtype males. SIT is most efficient if only males are released (Rendón et al., 2004). Preventing the introduction into the population of females that can damage fruit crops or transmit disease is an additional advantage. However, male-only releases require an efficient mechanism for sex sorting. For this purpose, genetic sexing strains (GSS- **Box 1**) were developed that differentiated between males and females with selective markers such as pupal colour or conditional lethality when exposed to high temperatures (Rendon, 1996).

First-generation transgenic systems sought to improve these technologies by creating analogues of GSSs that could also be used as population suppression measures in the field. The most widely adopted of these was the Release of Insects carrying a Dominant Lethal technology (RIDL) (Thomas et al., 2000), but also see (Schetelig and Handler, 2012) (Ogaugwu et al., 2013; Schetelig et al., 2016). The basis behind RIDL is the genetic modification of a pest to carry a deleterious/lethal gene whose expression can be turned off (repressed) during rearing, but which, when inherited by the progeny of released insects, will result in lethality for individuals in the field. As with SIT, mass releases of RIDL insects can thus suppress a target population by continually killing off field-born individuals before they can reproduce. Female-specific RIDL (fsRIDL) and genetic sexing strains have been developed in many species by combining this repressible lethality with sex-alternatively spliced introns from basal genes within the sex determination pathway (Fu et al., 2007; Schetelig and Handler, 2012; Ogaugwu et al., 2013; Tan et al., 2013), specifically *dsx* and *tra*. The pre-mRNA of these two genes is spliced differently between males and females—leading to the sex-specific inclusion or exclusion of exonic sequences. Sex-specific transgene expression can be designed by including the sequences responsible for sex-specific splicing (introns) embedded within components integral to the repressible-lethal system. As such, functional fsRIDL proteins are produced in one sex (usually females) and not the other (in the same manner as *tra* and sex-specific dsx proteins). Released fsRIDL individuals are homozygous fertile males that produce heterozygous male-only offspring when mating to wildtype females following release. These heterozygous fsRIDL males can then produce wildtype males and females as well as heterozygous fsRIDL males, resulting in a steadily diluting suppressive effect without continued releases. Female-specific RIDL lines have been developed in many insect pest species, including tephritid fruitflies (Fu et al., 2007; Ant et al., 2012), blow flies (Yan and Scott, 2020), screwworms (Concha et al., 2016), moths (Morrison et al., 2012; Jin et al., 2013) and mosquitoes (Phuc et al., 2007; Collado, 2013). Proof-of-principle demonstrations have also been made in beetles (Gregory, 2015). Caged and open-release trials have demonstrated that repeated releases of fsRIDL males can cause the rapid suppression of target populations (Harris et al., 2011; Leftwich et al., 2014; Carvalho et al., 2015; Shelton et al., 2020).

For these first-generation technologies, it is not necessary to know the precise means by which the sex-specific processing of *dsx* or *tra* components are regulated (i.e., the upstream elements of the pyramid which act upon them). All that is required is a basic understanding of the arrangement of the chosen gene and final mRNA differences between sexes. This conservation of function is a distinct advantage for adapting transgenic constructs, with minimal changes, across multiple species (Tan et al., 2013). A further advantage of using highly conserved basal elements of the pyramid is that the splicing signals which regulate their sex-specific splicing are often shared between closely related species (Ant et al., 2012). For example, an fsRIDL construct built using intronic sequences from pink bollworm (*Pectinophora gossypiella*) *dsx* functioned just as effectively in that species as it did in silkworm (*B. mori*) and diamondback moth (*Plutella xylostella*) (Jin et al., 2013; Tan et al., 2013). The limitation of using these downstream elements of the pyramid is that these systems are generally limited to *killing* females rather than their conversion to males. This, coupled with the self-limiting nature of these technologies, makes them far less potent than "second-generation" technologies (next section).

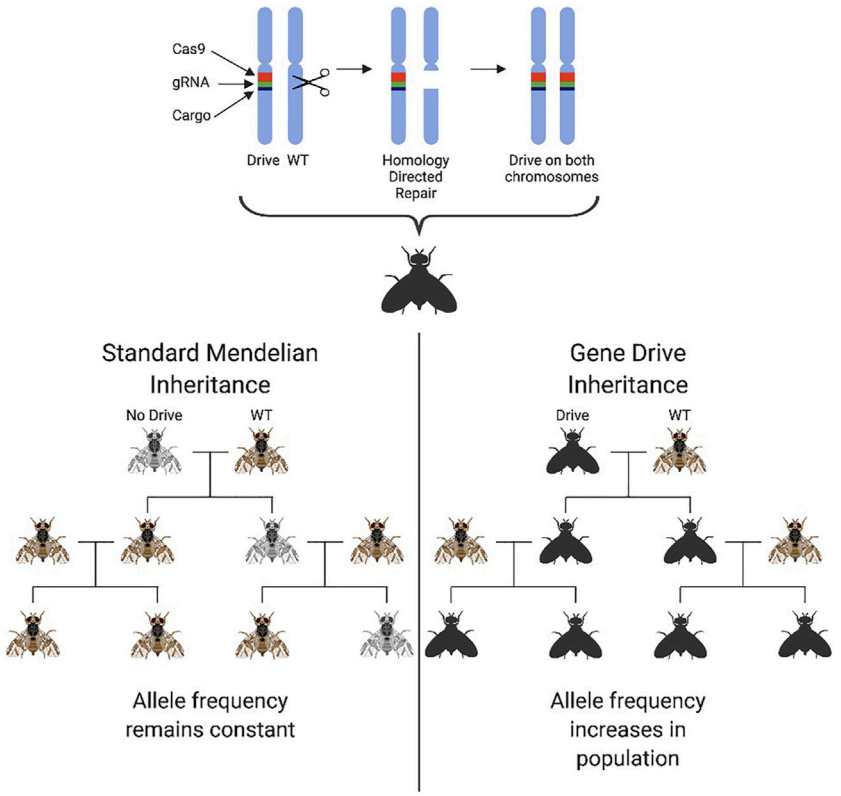

FIGURE 3 | The genetic principle and biased inheritance of a homing gene drive. Homing drives ensure their own transmission to the next generation through homology-directed repair. The Cas9 element of the construct, coupled with specific guides, cut the wildtype chromosome at a precise genomic location triggering a double-strand break. This break is then repaired using homology-directed repair with the remaining chromosome being used as a template. The drive construct is therefore present on both chromosome copies ensuring 100% offspring inheritance. A cargo element is also coupled with the drive elements which can spread a desired trait through a population. This biases the usual 50% inheritance mandated by standard Mendelian genetics and results in the 100% inheritance of the drive in offspring. The drive frequency in the population will therefore increase with subsequent populations as opposed to the standard mendelian inheritance at which the allele frequency would remain at a constant level in the population if a fitness cost is not incurred.

SECOND-GENERATION TECHNOLOGIES: CURRENT STATE-OF-THE ART

Gene Drive

Advances in genome editing, particularly the development of the CRISPR/Cas9 platform, have allowed a new generation of GPM technologies for population suppression to be developed. Commonly referred to as "homing gene drives" (HGD), these second-generation technologies were designed for population suppression or alteration (Burt, 2003; Alphey, 2014) and engineered initially using homing endonuclease genes and, more recently, CRISPR/Cas9. A gene drive is any system in which genes enhance their transmission in a sexually reproducing population above that predicted by Mendelian inheritance (50%). This enhanced transmission is beneficial in a pest control context as it allows "fitness-reducing traits", e.g., pesticide/environmental susceptibility or sterility, to be spread through a target population by the autonomous action of the gene drive. Homing gene drives encode a nuclease that recognises and cuts a sequence at the target wildtype locus on the homologous chromosome to where the HGD transgene is inserted. Double-stranded DNA breaks are known to be "editogenic," and under some conditions, cells can use intact homologous DNA as a template to repair the broken DNA. As such, during repair of this break, the broken strands are resected, and the host cell uses the intact HGD-bearing chromosome as a template to fill in the gaps, in a process known as homology-dependent repair (HDR). In using the HGD-bearing chromosome as a template for repair, this sequence copies itself onto the repaired chromosome. If this process happens efficiently and as part of the organism's germline, most gametes will receive a copy of the HGD (super-Mendelian inheritance). Depending on its imposed fitness costs, the gene drive element may then increase in frequency within a population. With a high transmission efficiency, theoretically extreme enough to spread throughout a population even at a fitness cost to the individual, gene drives have been seized upon by those working on genetic methods for pest control for their ability to engineer populations even at low introduction frequencies (Gantz and Bier, 2015; Gantz et al., 2015; Hammond et al., 2016; Champer et al., 2018) **Figure 3**. Currently one of the most pressing issues in the "gene-drive" community is the control and safe practice of potential gene drive

releases, leading for example, to the development of confinable split-drive technologies that also safeguard against accidental release (Li et al., 2020; López Del Amo et al., 2020).

HGDs designed for population suppression are inserted into (and therefore disrupt) a gene of essential function. If that essential gene is haplosufficient, the HGD causes a deleterious recessive phenotype. Heterozygous individuals are viable and contribute to the spread of the drive, but when the drive reaches a high allele frequency in the population, more and more non-viable homozygous individuals are produced, leading to a dramatic reduction in the reproductive capacity of the population (Deredec et al., 2011). The most efficient designs target viability in only one sex (usually females), allowing the drive to spread efficiently within the other sex, regardless of frequency.

As with any pest elimination strategy, selection for resistance to a gene drive is a concern. The most commonly cited mode of resistance is mutations in the target cut sites "resistance alleles," which prevent further recognition by the Cas9/sgRNA complex and therefore targeting by the drive. If resistance alleles do not severely disrupt the gene's coding potential (e.g., synonymous mutations, or small in-frame deletions), they may be rapidly selected for in a drive carrying population as the fitness differential between the resistance mutation and the drive is expected to be large (Champer et al., 2017; Carrami et al., 2018). Several strategies can mitigate this, including restricting expression of the gene drive to the germline (Hammond et al., 2021) targeting highly conserved gene sites, hoping that this indicates low tolerance for mutated alleles. In at least one example in *A. gambiae,* this appears to have been achieved by targeting the intron 4 - exon 5 boundary (splice junction) of *dsx* (Kyrou et al., 2018). Typically, this fifth exon is included in female, but not in male, *dsx* mRNA (female-specific exon). However, when the splice junction is disrupted, the 5th exon is instead excluded (skipped) in both sexes. Homozygous deletions at this junction incapacitated female sexual development leading to intersex and sterile females as these individuals could not produce functional dsxF (lacked the 5th exon). Male development and fertility, however, was unaffected as this 5th exon is canonically excluded from the final mRNA transcript in that sex. Within this study, this produced a highly effective drive, spreading female non-viability through a caged population, leading to a rapid population crash (Kyrou et al., 2018).

Much like earlier "first-generation" systems, the use of highly conserved, downstream basal elements of the sex determination pathway as essential components of a suppression drive produced reliable, predictable and effective mechanisms of generating female non-viability (in this case sterility, rather than lethality, as with first-generation technologies). The highly conserved and well-understood role of *dsx* in the downstream regulation of female sexual development means that it is highly likely that similar suppression drives could be developed in a range of other insect pest species. However, there are differences between the number and "style" of exon skipping which occurs between different insect species, which would require consideration.

While no functional resistant alleles were observed in this study, it is possible that at larger-scale releases, pre-existing or *de novo* alleles might eventually occur (Bier, 2021). Including multiple guide RNAs designed against numerous sequences at the target loci, also known as "multiplexing," is one frequently discussed mitigation against this (Carrami et al., 2018). Pre-existing sequence variations or failed homing attempts must inhibit all target sequences simultaneously to inhibit the drive and are therefore less likely to generate functional resistant mutants (Marshall et al., 2017; Champer et al., 2018, 2020b; Oberhofer et al., 2018; Champer S. E. et al., 2020). However, the small target site of the exon-splice junction in *dsx* means that multiplexing guide RNAs would be difficult to engineer for this gene and would likely be an issue in using homologous targets in other species. Complementary alternatives, including combinate X-shredder drives, have been demonstrated (Simoni et al., 2020).

Precision-Guided Sterile Insect Technique

One alternative approach to using CRISPR/Cas9 to develop HEGs is to improve "first-generation" technologies with precise gene editing. Coupling the precision of CRISPR/Cas9 gene editing to enhance SIT has been proposed and developed as pgSIT (precision-guided sterile insect technique). This alternative approach to using CRISPR/Cas9 creates sterile males and kills or incapacitates females by targeting both a male fertility gene such as *beta2-tubulin* and elements of the sex determination pathway such as *dsx, tra* or *sxl.* RNA guides targeting *sxl* and *beta2-tubulin* coupled with a *Cas9* under a germline-specific promoter killed female embryos and produced sterile, male-only progeny in *Drosophila.* Targeted knock-out of *dsx and tra* resulted in intersex females (Kandul et al., 2019). Newer developments include a temperature inducible true-breeding strain that eliminates the requirement of maintenance and sexing of two independent parental strains (Cas9 and gRNA) (Kandul et al., 2021) Like suppression gene drives, the downstream, basal elements of the sex determination pathway are a reliable target for female non-viability. Versions of pgSIT have also been developed in mosquitoes (*Ae. aegypti*), and unlike a gene drive, this approach is self-limiting and is not predicted to persist or spread in the environment (Li et al., 2021).

THIRD-GENERATION TECHNOLOGIES: LOOKING TO THE FUTURE

Whereas first- and second-generation technologies seek to manipulate or disrupt basal elements of the sex determination pyramid in order to reduce the fitness of or kill females, future "third-generation" technologies may be designed to manipulate the master regulators of sexual fate, to affect full sex conversion. If attached to an efficient gene drive system, such a technology would spread through a target population causing a growing wave of sex distortion. This is theoretically more efficient than a second-generation system that kills or incapacitiates one sex as homozygotes, because all inheritors, regardless of their genotype, continue to spread the system. This increased efficiency could potentially allow for a dramatic reduction in the number and size of releases required for population control. Depending on the efficiency of sex conversion, this could enable threshold-dependent gene drives to be used, previously discounted for population suppression because of their intolerance to high fitness loads (Leftwich et al., 2018; Champer et al., 2020a).

Many aspects of the theory underpinning such third-generation technologies pre-date second-generation strategies (Lyttle, 1991). However, they have proven challenging to enable in practical terms. Part of the reason is the non-conserved nature of the upstream components that need to be manipulated. This requires deep and specific knowledge of master regulators and their web of interactions with downstream elements for each specific pest species to be targeted. Even then, the unpredictable/inflexible nature of "coupled" sex determination and dosage compensation systems may make such a design unachievable in some species. Hence for third generation systems, the transfer of efficient gene drive designs between pests may not always be possible. In a similar vein, if sex-specific components of fitness are sex-linked it may be the case that efficient sex conversion can be achieved, but the sexual competitiveness of converted individuals is diminished.

The mechanisms for enacting sex conversion will vary greatly depending on whether the targeted gene(s) are the master regulators of sex determination (and whether these initiate a male or female cascade), or those genes directly downstream (*tra* or *tra-2*). In an XX/XY system, sex conversion through expression of a master regulator is likely to produce a dominant effect. This is highly likely to affect the dynamics of a gene drive. For example, the effects on population suppression would be seen much earlier than one where conversion is enacted through disruption of a recessive switch e.g., *tra* or *tra-2* (Hoshijima et al., 1991; Pane et al., 2002; Sarno et al., 2010). For systems where the presence of the master regulator determines femaleness [for example, the ZZ/ZW systems common to Lepidoptera (Suzuki et al., 2010)], maleness could be achieved by inactivating the master regulator or making the element below it resistant to its activity [see (Sakai et al., 2016)].

In the next section, we discuss evolutionary and empirical manipulation studies of dosage compensation and sex-linked fitness traits and outline the hurdles these may pose to engineering efficient third-generation technologies.

Dosage Compensation

In heterogametic sex chromosome systems, the loss of recombination between the dissimilar chromosomes leads to multiple evolutionary processes acting to reduce the size of the sex-limited chromosome, including mutation accumulation and gene loss (Bachtrog, 2013; Bachtrog et al., 2019). This can lead to a monoallelic state for the heterogametic sex, in which a single functional allele is present for multiple genes on the single copy of the X or Z chromosome, and the homogametic sex retains two functional copies. This imbalance of alleles between the sexes is often hypothesised to require dosage compensation mechanisms to restore a balanced state of gene expression: classically, this was thought to occur across the entirety of the X or Z chromosome (Ohno, 1967). If dosage compensation occurs across the entirety of sex chromosomes in a target pest species, it could prove challenging for the design of third-generation technologies, particularly if the dosage compensation pathway is downstream of the master regulator (i.e., the pathways are "coupled"). This is because, while such a system would ectopically express a sex determination master regulator, it would not alter the sex chromosome complement of an individual. If the two pathways are "coupled", that individual (say, a female) would enact the dosage compensation pathway of the opposite sex (a male), despite having a "female" sex chromosome complement. This would lead to lethal misexpression of sex-linked genes and death, rather than conversion to the opposite sex.

Fortunately for GPM engineers, there is growing evidence from evolutionary studies of an alternative model of gene-by-gene dosage compensation. This alternative model states that only a minority of loci may be dosage-sensitive, specifically genes with particularly high expression levels, or those that have evolved through recent gene duplication. This may have a low correlation with levels of observed sex chromosome divergence (Furman et al., 2020). Where global dosage compensation is primarily observed is in XY systems, and could be driven by the stronger sexual selection and greater reproductive variance in males, this is predicted to result in slower evolution of Z than with X dosage compensation (Mullon et al., 2015). This could mean that sex chromosome dosage compensation may be less of a challenge in ZZ/ZW systems such as Lepidoptera (Gu et al., 2017).

In reality, the nature of dosage compensation appears to vary widely, and exceptions to "general" rules seem to be increasingly common. For example, *A. gambiae* has an XY heterogametic sex-determination system, with a single gene, *Yob,* identified as a Y-linked maleness factor (Krzywinska et al., 2016). The expression of *Yob* begins around 2 hours into embryonic development and precedes that of sex-specific splicing of *dsx* by about 6 hours. Ectopic expression of *Yob* has been confirmed to produce male splice-form *dsx* but leads to female embryonic death while leaving male development unaltered (Krzywinska and Krzywinski, 2018). This pattern of female lethality in the presence of *Yob* can be explained by gene overexpression by both X chromosomes as a result of misapplied dosage compensation leading to female death. Similar experiments in the mediterranean fruit fly (*C.capitata*), which also has an XY heterogametic sex-determination system, have also identified a Y-linked single gene determinant of maleness, *MoY*. Here knockdown of *MoY* was demonstrated to be sufficient to produce total loss of male-specific *tra* mRNA in embryos and complete XY feminisation. Conversely, ectopic expression of *MoY* produced no change in male development and partial or full masculinisation of XX flies (Meccariello et al., 2019). These XX pseudomales were also fertile, demonstrating that there are no genes *essential* to male fertility located on the Y chromosome in medfly. RNAi knockdown of *tra* in several other tephritid fruitflies and *M. domestica* have also produced female to male sex reversal, producing fertile converted males, indicating this approach may be possible in a number of pest insects (Dübendorfer et al., 2002; Pane et al., 2002; Lagos et al., 2007; Concha and Scott, 2009; Hediger et al., 2010; Schetelig et al., 2012) We note though the genetic factors that may influence the outcome of sperm competition have not yet been studied in these systems. In both *An. gambiae* and *C. capitata*; closely related species (*Anopheles arabiensis* with *Yob*; *Bactrocera oleae* with *MoY*), appear to be responsive to their respective male determining signals. However, the fast-evolving nature of the primary sex determination regulators means that these are likely to be restricted to closely related species with either direct homology to these genes or conserved downstream

BOX 1 | Alternative methods to altering sex ratios outside of the sex determination pathway

Two modifications are principally required for Genetic Sexing Strains (GSS) 1) introduction of a recessive conditional lethal gene or viable selectable recessive colour mutations and 2) translocation of a wild-type rescue allele onto the male Y-chromosome. In the final strains, females are homozygous for one or more selectable mutations, while males are heterozygous with a wildtype phenotype (Rendon, 1996). These strains are highly effective at producing substantial bias in the reproductive sex ratios or enabling efficient sex separation, but because of the mutations and chromosome translocations required to generate these strains, high levels of sterility and rearing difficulties were common, many strains were also unstable as a result of these complex chromosomal rearrangements (Robinson, 2002; Nguyen et al., 2021).

Genetic sexing strains produced strong genetic male bias, achieved not through manipulation of the sex determination pathway, but by positioning autosomally lethal alleles onto the sex chromosomes. The advent of powerful genome editing tools and synthetic biology has allowed for the development of other, more refined artificial sex distortions such as X-shredding. These systems exploit the heterogametic nature of these species, where fathers always transmit their X chromosome to their daughters and the Y chromosome to their sons, to cause lethal changes to essential genes, without involving the sex determination pathways directly.

X-shredders were first pioneered in *Anopheles gambiae* when I-PpoI was discovered to cut the X chromosome in several locations due to its targeting of the repeated ribosomal rDNA (Windbichler et al., 2007). By engineering a destabilised version I-PpoI its activity could be restricted to male meiosis thereby ensuring males were unable to pass on a functional X chromosome (Galizi et al., 2014). As opposed to using an endonuclease which targets a conserved repetitive element; X-shredding can also be driven using CRISPR/Cas9 and targeted sgRNAs, with Cas9 cleavage limited to the male germline (Galizi et al., 2016; Fasulo et al., 2020; Meccariello et al., 2021).

Y-linked editors have also been proposed as a self-limiting strategy significantly more effective than those previously proposed (Burt and Deredec, 2018). If released simultaneously with an autosomal X-shredder this efficiency can be further increased. An alternative approach would be to drive an X-shredder from the Y-chromosome to ensure male offspring inheritance (Gamez et al., 2021). Other proposed Y-linked suppression systems include Medusa; combining a maternally-expressed, X-linked toxin and a zygotically-expressed, Y-linked antidote that causes suppression of the female population and selects for the transgene-bearing Y. At the same time, a zygotically-expressed, Y-linked toxin and a zygotically-expressed, X-linked antidote selects for the transgene-bearing X in the presence of the transgene-bearing Y to create a threshold dependent, highly male-biasing suppression system (Marshall and Hay, 2014) present its own challenges as expression during spermatogenesis can be difficult to achieve from the Y chromosome due to transcriptional repression (Alcalay et al., 2021).

interactions. The disparate responses of female death vs. female-male sex conversion between these two species were entirely unpredictable, and while the role of dosage compensation in this is speculative (and does not preclude that a dosage compensation mechanism exists that is uncoupled from sex determination); evidence to-date indicates these different fates for alteration of sexual development could be the result of just a handful of dosage-sensitive genes on the X chromosome of *An. gambiae* while none are present on the X chromosome of the medfly.

Essential Male Genes

One prediction of the evolution towards heterogametic sex chromosome systems is the accumulation of sex-specific fitness-enhancing genes on the sex-specific region, often linked to the master regulator through lack of recombination (Mank et al., 2014). As with dosage compensation, this arrangement may prove a hurdle for third-generation sex-conversion systems if these fitness-enhancing genes are not included alongside the master regulator. The Y chromosome of *D. melanogaster* contains male fertility factors. However, it contains only 16 protein-coding genes, and not all are essential for male fertility (Kaiser and Bachtrog, 2010; Zhang et al., 2020). Other examples of essential genes in males can be seen with the engineered manipulation of the male master regulator in *Ae. aegypti* and *Ae. albopictus*. These two mosquitos do not possess hetermorphic sex chromosomes, only a small, non-recombining male-determining region (M-locus) on the short arm of chromosome 1, an otherwise homomorphic autosome (Hall et al., 2014). A single gene *Nix* [a putative recent duplication of *tra2* (Gomulski et al., 2018)], has been identified as the master male determining gene in these species (Liu et al., 2019; Aryan et al., 2020). In *Ae. aegypti*, stable transgenic expression of *Nix* was sufficient to produce sex conversion of genotypic females into males. Dosage compensation in a species without a heterogametic sex would be unlikely, and the observed sex conversion over female death was in line with this prediction. However, while *Nix* was sufficient for determining male sexual fate, the resultant pseudomales were incapable of flight as they lacked another gene *myo-sex*, also present in the M-locus, which is required for proper development of flight muscles in males (Aryan et al., 2020). Conversely, when analogous experiments were conducted in *Ae. albopictus* it was found that converted pseudomales were not only viable but capable of flight (Lutrat et al., 2022). Interestingly, despite evidence of an M-linked *myo-sex* gene, converted pseudomales could still express comparable levels of *myo-sex* transcripts to wildtype males. These results indicated at least one duplicate copy of *myo-sex* exists which is not M-linked in this species. These converted pseudomales, displayed reduced competitiveness compared to wildtype males, suggesting the possibility that the M-locus in this species may harbour other, as yet unknown, male fitness-enhancing genes. However, this is difficult to disentangle from the adverse effects of transgenesis (including ubiquitous marker gene expression, disruption of essential genes at the insertion sites or incomplete masculinization). These findings highlight that, even in species without apparent dosage compensation or heteromorphic sex chromosomes, efficient sex conversion technologies may prove more challenging to engineer than simply transgenically expressing the master regulator. Additionally, the significant differences between these two closely related species suggest that substantial fundamental research will be required to underpin the development of these technologies in novel pests.

CONCLUSION

Manipulating sex determination pathways for genetic pest management has many potential applications. Previous technologies have used the highly conserved "basal" elements of *dsx* and *tra* common to almost all insect species to produce reliable mechanisms of biasing sex ratios with the release of modified males carrying factors to generate female sterility or death. Newer

technological developments, including homing gene drives, demonstrate these basal elements continue to be predictable and reliable targets for control. Looking forward, development of genetic editing techniques to manipulate "master regulators" of sexual fate have the potential to improve the performance of a wide variety of genetic control methods. However, this approach has potential challenges—different species may exhibit sex-linked genes that are vital for viability or sexual fertility or have strong dosage compensation. However, this is a vibrant field of research and much experimental work is ongoing in a range of different pest species. While it is likely that the application of sex conversion for pest control will inevitably be applied on a case-by-case basis, active investigations on a number of fronts are likely to improve our understanding of the basic biology and evolution of sex determination, as well as expand our genetic toolbox for applied pest management.

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Standardization of the FAO/IAEA Flight Test for Quality Control of Sterile Mosquitoes

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Successful implementation of the sterile insect technique (SIT) against *Aedes aegypti and Aedes albopictus* relies on maintaining a consistent release of high-quality sterile males. Affordable, rapid, practical quality control tools based on the male's flight ability (ability to escape from a flight device) may contribute to meeting this requirement. Therefore, this study aims to standardize the use of the original FAO/IAEA rapid quality control flight test device (FTD) (version 1.0), while improving handling conditions and reducing the device's overall cost by assessing factors that could impact the subsequent flight ability of *Aedes* mosquitoes. The new FTD (version 1.1) is easier to use. The most important factors affecting escape rates were found to be tube color (or "shade"), the combined use of a lure and fan, mosquito species, and mosquito age and density (25; 50; 75; 100 males). Other factors measured but found to be less important were the duration of the test (30, 60, 90, 120 min), fan speed (normal 3000 rpm vs. high 6000 rpm), and mosquito strain origin. In addition, a cheaper version of the FTD (version 2.0) that holds eight individual tubes instead of 40 was designed and successfully validated against the new FTD (version 1.1). It was sensitive enough to distinguish between the effects of cold stress and high irradiation dose. Therefore, the eight-tube FTD may be used to assess *Aedes'* flight ability. This study demonstrated that the new designs (versions 1.1 and 2.0) of the FTD could be used for standard routine quality assessments of *Aedes* mosquitoes required for an SIT and other male release-based programs.

Keywords: mass-rearing, sterile insect technique, *Aedes aegypti*, *Aedes albopictus*, age, cost, color

1 INTRODUCTION

The mosquito is one of the world's deadliest animals. The Asian tiger mosquito *Aedes albopictus* (Skuse) , together with *Aedes aegypti* (Linnaeus) are highly invasive (Benedict et al., 2007) and medically important mosquito species that transmit several arboviruses and associated diseases including dengue, chikungunya, yellow fever, and Zika (Bhatt et al., 2013; Levy Blitchtein and Del Valle Mendoza, 2016).

There are a limited number of effective vaccines or drugs to protect against these diseases; thus, current disease control strategies rely on Aedes vector control, where the focus is usually on source reduction and the use of insecticides. However, the variability in the type of *Aedes* larval breeding sites and the presence of a myriad of cryptic breeding habitats coupled with the spread of insecticide resistance (Moyes et al., 2017) has created an even greater global need for alternative tools to control these disease vectors. Researchers have advocated for genetic control strategies, including the sterile insect technique (SIT) as an environment-friendly, insecticide-free technique. The SIT is based on inundated and repeated releases of sterile insects to induce sterility in the wild population to suppress the target pest species (Knipling et al., 1968). Great progress has been achieved over two decades in the development of the SIT package for mosquitoes. The Insect Pest Control Laboratory (IPCL) of the joint Food and Agriculture Organization/ International Atomic Energy Agency (FAO/IAEA) Centre of Nuclear Techniques in Food and Agriculture has been leading the development of methods and guidelines, including mass-rearing equipment, irradiation, packing, transport, quality control and release for *Anopheles* and *Aedes* mosquitoes for deployment in field projects in member states reviewed in Vreysen et al. (2021).

Historical data have shown the achievements of SIT against several pests and vectors (Dyck et al., 2021). However, Benedict and Robinson (2003) have shown that several trials conducted in the 1970s had limited success due to the poor competitiveness of released males. Therefore, the quality of released males is critical to the success of SIT within an area-wide integrated pest management program (Parker et al., 2021). Several factors, including colonization, mass-rearing, irradiation, handling, and releases, may affect the quality of males in terms of their capacity to disperse, survive, locate females, and compete for mates (Bouyer and Vreysen, 2020; Parker et al., 2021). Important factors that impact the quality of male mosquitoes such as flight performance can only be assessed by time-consuming methods such as male mating competitiveness assay in the laboratory and mark–release–recapture (MRR) in the field. In addition, MRR experiments provide low recapture rates (Bellini et al., 2010; Bouyer et al., 2020; Iyaloo et al., 2020) and are tedious and costly, while conventional mating competitiveness assay is not only laborious but time-consuming. To circumvent these difficulties, a quick, easy-to-perform, and reliable quality control (QC) method to use before adult mosquitoes are released would be highly beneficial.

Flight is one of the most important traits of mosquito life history. Flight ability (capacity to escape or to fly) has been shown as a critical QC factor in fruit fly factories. Understanding this factor has helped to reduce production costs by improving rearing techniques in Mediterranean fruit fly mass-rearing facilities and has improved the level of competitiveness in released males (Shelly et al., 2010). Rowley et al. (1968) designed a flight mill, and other authors developed a video tracking system (Boyer et al., 2013; Lebon et al., 2018) to measure insect flight speed and the distance flown. Recent studies have developed mosquito QC tools based on a mosquito's ability to escape from a flight test device (FTD). While some of the devices developed were based on young adults that had directly emerged from pupae (Balestrino et al., 2017), others used two-to-three-day-old adult mosquitoes (Culbert et al., 2018; Dor et al., 2020). Modified gray-colored polyvinyl chloride (PVC) flight tubes with different diameters and heights were tested to ascertain whether mosquito age or the height of the FTD would impact male *Ae. aegypti* escape rates (Dor et al., 2020). These modifications resulted in higher escape rates in all cases after 24 h. The FAO/IAEA reference QC FTD has been successfully tested in terms of assessing the flight performance of irradiated males and males under cold stress conditions after 2 hours (Culbert et al., 2018). Although preliminary tests were performed to design the FTD prototype (Culbert et al., 2018), further tests are needed in order to fine-tune the FTD for improved sensitivity and accuracy. These further tests include assessing the role of fan speed (airflow), the test duration, the density of males, the color of the flight tubes, the lure [Biogents (BG) pellets], fan or the presence of fan, lure, the mosquito strain origin, and the number of flight tubes within the device. In addition, the number of mosquito SIT programs is growing (Bouyer et al., 2020; WHO and IAEA, 2020), and facilities are in need of a standardized and efficient QC test. Several research groups have tested the prototype FTD and found inconsistent results and difficulties running the test. Therefore, this study aims to modify the original FTD to measure its efficiency while improving handling operation and test sensitivity and repeatability, while reducing its overall production cost. Factors in the test design that could affect the flight test's applicability were investigated in order to standardize the device and user protocol.

2 MATERIALS AND METHODS

2.1 Mosquito Strains, Rearing, Irradiation, and Cold Stress Conditions

Standard laboratory reference strains of *Ae. aegypti* and *Ae. albopictus* (FAO/IAEA, 2017, 2020) were used for all experiments. The *Aedes* strains were maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* Mosquitoes" (FAO/IAEA, 2017) (*Experiments 2.3 to 2.9*). *Aedes aegypti* and *Ae. albopictus* strains originating from Brazil (Juazeiro) and Italy (Rimini) were transferred to the IPCL from the insectary of Biofabrica Moscamed, Juazeiro, Brazil, and from the Centro Agricoltura Ambiente, Bologna, Italy, in 2012 and 2018, respectively. These two institutions are IAEA collaborating centers for the development of mosquito SIT. These strains were used to assess factors that might affect mosquito flight ability (*Experiments 2.3–2.9*). Two recently colonized *Ae. albopictus* strains originating from Spain (Valencia, TRAGSA) and China (Guangzhou, Wolbaki) were used to assess flight ability at the IPCL (*Experiment 2.9*). In addition, a *w*AlbB strain of *Wolbachia*-infected *Ae. aegypti* (hereafter referred to as the "Singapore strain") was independently maintained (Cheong Huat Tan, personal communication) at the Environmental Health Institute of the National Environment Agency, Singapore, to replicate two sets of standardization experiments (*Experiments 2.6–2.7*).

The rearing period had controlled conditions as follows: temperature of 28 ± 2°C, 80 ± 10% relative humidity (RH), and lighting of 14:10 h light: dark, including 1 h of dawn lighting and 1 h of dusk lighting for larval stages. Adults were separately maintained under 26 ± 2°C, 60 ± 10% RH, and 14:10 h light: dark, including 1 h dawn and 1 h dusk.

To perform the experiments, mosquitoes were reared following modified mass-rearing procedures developed at the IPCL (Mamai et al., 2019; Maïga et al., 2019; FAO/IAEA, 2020). Larval rearing started on Thursdays (day zero) when eggs were hatched and transferred to mass-rearing trays previously filled with 5 L of osmosis water on Fridays (day one). No larval feeding was performed during weekends and pupae were collected on day six after egg hatching. A 4% IAEA larval diet was provided daily: a 300 ml (0.66 mg/larva) meal on day one, a 300 ml (0.66 mg/larva) meal on day four, and a 200 ml (0.44 mg/larva) meal on day five. Larvae and pupae were separated on day six. Four liters of larval water were reused with an additional 300 ml for day six of larval rearing. Pupae were collected daily and sex-separated using mechanical and semiautomatic pupal sex sorters (John W. Hock Co., Gainesville, FL; Wolbaki, China).

For each experiment, pupae were aliquoted into 100 ml plastic cups (Medi-Inn, United Kingdom), each holding 110 male pupae and placed in cages (15 × 15 × 15 cm, BugDorm, BD4M1515, Taiwan) for emergence. About 100 (accounting for emergence and mortality rates) adults were maintained with access to a 10% sucrose solution until the day of the experiments.

To assess the sensitivity of the new FTD (version 2.0) (*Experiment 2.9*), three-to-four–day-old adult *Aedes* mosquitoes were exposed to cold stress conditions (4°C for 2 hours), which is known to significantly impact the flight ability of both *Ae. aegypti* and *Ae. albopictus* (Culbert et al., 2018). Males were allowed to recover for 2 h in the presence of a 10% sucrose solution prior to the test.

To assess the sensitivity of the new FTD (version 2.0) (*Experiment 2.9*), three-to-four–day-old adults were exposed to high-dose irradiation (100 Gy using an X-ray blood irradiator (Raycell MK2) (Gómez-Simuta et al., 2021) for *Ae. albopictus* and 150 Gy using the GammaCell 220 (Nordion Ltd., Kanata, Ontario, Canada) for *Ae. aegypti*), which is known to reduce the quality of mosquitoes (Culbert et al., 2018). Males were knocked down and held in a cold room at 4°C in compacted batches of 100/cm^3 to simulate mass-transport conditions prior to irradiation. Males were allowed to recover for 2 h with a 10% sucrose solution prior to the flight ability test. Untreated (nonirradiated) male mosquitoes were kept in laboratory conditions with a 10% sucrose solution during both the cold stress and the irradiation treatments.

The test conditions for all experiments were 26 ± 2°C and 60 ± 10% RH, under a laboratory daylight regime (500–1000 lux), using untreated male mosquitoes (unless stated otherwise).

2.2 Modification of the Original Flight Test Device and Operation

The original FTD (Culbert et al., 2018) (version 1.0) was modified in several ways to measure its efficiency while improving handling processes. The original FTD consists of 40 transparent acrylic plastic (polymethyl methacrylate, PMMA) tubes (hereafter referred to as "individual internal tubes") that were placed together within a larger PMMA tube (hereafter referred to as the "inner tube"). Gaps between individual internal tubes were filled with transparent silicone (OBI, Austria). When male mosquitoes escape from the flight device, they can be recollected in a larger cylindrical PMMA tube (hereafter referred to as the "containment box"), which is closed at the top end with mesh (**Figure 1A** and **Supplementary Material S1**). A fan (DC axial fan: 40 mm, Vapo: 12 V, airflow: 0.218 m^3/min, acoustic noise: 20.6 dB, and rated speed: 6,000 rpm, Multicomp, United Kingdom) and a BG-Lure (Biogents, Regensburg, Germany) pellets holder made of PMMA is placed on top of the end mesh.

To operate, the inner tube was inserted and removed from the bottom of the containment box, which was secured with a mesh sleeve. Mosquitoes were first loaded into the FTD before the device was introduced through the bottom in the larger cylindrical tube because the side opening was too small to allow side introduction. In addition, after the test was completed, the device was taken to a cold room to knock down the mosquitoes prior to counting. These handling processes were found to be tedious for nonexperienced operators and may have increased the risk of damaging the FTD. Therefore, we designed a square containment box to replace the larger cylindrical tube. The containment part of the device is easily opened from the top, and the inner tube can be inserted or removed more easily through a larger opening on the front side of the containment box [**Figure 1B**, **Figure 2** (version 1.1)].

In addition, another version of the FTD was developed. The new FTD (version 2.0) (**Figure 1C**) is made of eight individual internal tubes fitted in an inner tube of 4 cm in diameter. The tube holder (base) was resized to fit the inner tube better. The containment box and the tube length are all similar to version 1.1.

For all experiments, the following steps were followed to perform the flight test (**Supplementary Materials S13, S14**). Before any test, one to two pellets (10–30 mg) of BG-Lure (Biogents, Regensburg, Germany) were placed on top of the FTD. A fan was placed directly on the small BG decoy cap and switched on at its standard speed (3000 rpm unless stated otherwise). About 100 adult male mosquitoes (unless stated otherwise) were aspirated using a mouth aspirator and were blown into the FTD *via* a small 1 cm hole at the bottom of the device. Males were then confined within a small space (height × diameter: 1 × 7 cm) and flew upwards through one of the individual internal tubes and out into the large containment box. After 2 hours, the top of the inner tube was covered using a Petri dish (9 cm in diameter) to avoid further escapes, the fan was stopped, and the experiment was deemed as complete. The inner tube was then removed through the large opening of the containment box by tilting it slowly. When tilted halfway, the Petri dish covering the top of the tube was held to avoid losing it. To remove the nonescaped males from the inner tube, mosquitoes were blown through the bottom into a cage

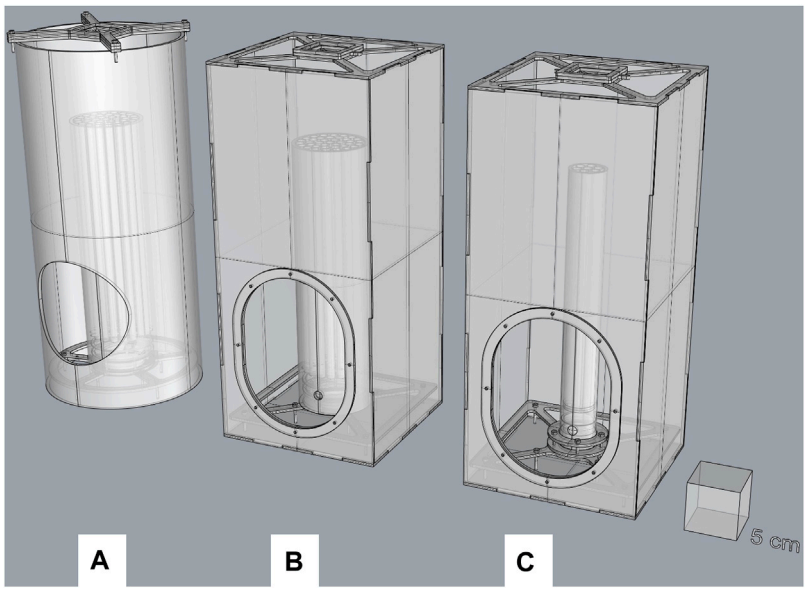

FIGURE 1 | 3D designs of the 40-tube original (version 1.0) **(A)** vs. the new FTDs: 40-tube FTD (version 1.1) in the middle **(B)**; eight-tube FTD (version 2.0) on the right **(C)**.

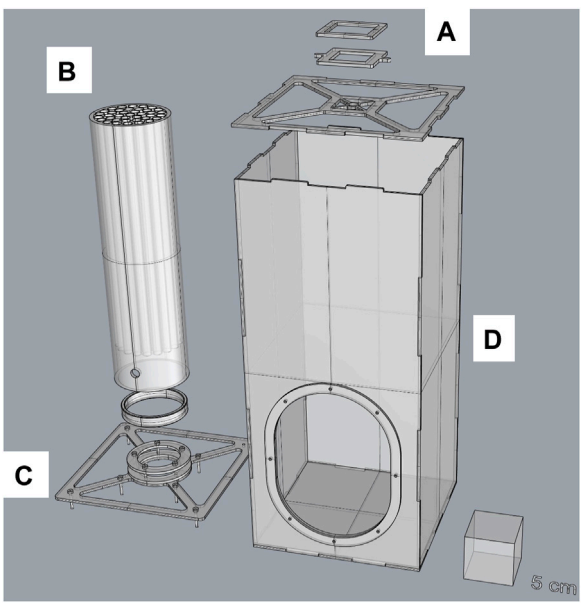

FIGURE 2 | Split design of the new 40-tube flight test device (version 1.1). The device comprises a top cover **(A)** that can hold a fan and BG-Lure pellets, a flight test system (inner tube) **(B)** with 40 individual internal tubes, an inner tube holder (base) **(C)**, and the main container (containment box) **(D)**. More details are provided in the technical drawings (**Supplementary Materials S2–S12**).

(15 × 15 × 15 cm, BugDorm, BD4M1515, Taiwan). The escaped males were removed using a mouth (or mechanical) aspirator from the containment box and transferred to another cage (15 × 15 × 15 cm, BugDorm, BD4M1515, Taiwan). All cages were taken into a freezer (−20°C) for 20–30 min and numbers were counted for each treatment.

2.3 Effects of Fan Speed or Airflow on Escape Rate

To assess whether the fan speed could affect a mosquito's capacity to escape, two fan speeds were tested. The FTD fan (Multicamp, United Kingdom) was set to either its highest speed (high, 6000 rpm) or at its standard speed (normal, 3000 rpm). Three FTDs (version 1.1) were tested with two-to-three–day-old male *Ae. aegypti* (Brazil strain) mosquitoes and four FTDs with four-to-five–day-old male *Ae. albopictus* (Rimini, strain) mosquitoes for each speed, respectively.

2.4 Effects of Test Duration on Escape Rate

It was initially observed that as males were being loaded into the FTD, a large number of mosquitoes escaped immediately. Therefore, to ascertain a suitable length of time to perform the QC flight test, a range of times were tested—including 30, 60, 90, and 120 min. The test was repeated four times (with two, two, two, and three pseudoreplicates per repetition, respectively) with two-to-three–day-old male *Ae. aegypti* (Brazil strain) and twice (two pseudoreplicates each) with two-to-three–day-old male *Ae. albopictus* (Rimini, strain) for each duration.

2.5 Effects of Male Density on Escape Rate

Densities of 25, 50, 75, and 100 males were tested to see whether the number of males loaded into an FTD would affect the output of the escape rate. The test was performed with four FTDs for each density with two-to-three–day-old male *Ae. aegypti* (Brazil strain) mosquitoes. For *Ae. albopictus* (Rimini strain)

mosquitoes, the density effect was assessed twice (four and two pseudoreplicates) with two-to-three–day-old males for each density.

2.6 Effects of Internal Tube Color and Addition of Lure and Fan on Escape Rate of *Aedes aegypti* and *Aedes albopictus*

To assess whether the color of the FTD could affect escape rates, two types of inner tubes (where the tube ends with transparent and pink silicon) were used (**Supplementary Material S15**). For each tube type (transparent and pink), the presence/absence of fan and lure was tested for *Ae. aegypti* (Brazil strain) and *Ae. albopictus* (Italy strain): one with the addition of BG-Lure only (pink/transparent + lure), one with fan only (pink/transparent + fan), one with fan and lure together (pink/transparent + fan + lure), and one FTD without a fan or lure (pink/transparent). The test was performed twice (four and two pseudoreplicates/treatment) with two-to-three–day-old male *Ae. aegypti* and with two-to-three–day-old male *Ae. albopictus* mosquitoes, respectively.

The same experiment was carried out with five-to-six–day-old male *Aedes aegypti* in Singapore (Singapore strain) using only the transparent FTDs (version 1.1). The escape rates from two FTDs with fan and lure (transparent + fan + lure) were compared to three FTDs of each of the following: with lure only (transparent + lure), with fan only (transparent + fan), and without fan and lure (transparent).

For the FTD to be tested without a fan (absence of a working fan), a fan was still placed on top of the device but without turning it on.

2.7 Effects of Male Adult Age on Flight Ability

The effect of age on male flight ability was assessed for different age groups ranging from less than 1 day up to 8 days old for *Ae. aegypti* (three replicates/age group) and from less than 1 day up to 10 days for *Ae. albopictus*. In addition, two age groups (12–13 and 16–17 days) were assessed for the latter. Four replicates were performed for each *Ae. albopictus* age group.

The same experiment was repeated at the Environmental Health Institute, National Environment Agency, Singapore. Male *Ae. aegypti* (Singapore strain), aged between two to six days, were tested. Four age groups, including two to three, three to four, four to five, and five to SIX days, were used to assess and compare mosquitoes' ability to fly using the 40-tube transparent FTD (version 1.1). The flight test was performed in total 21, nine, nine, and 27 times for the two-to-three-, three-to-four–, four-to-five–, and five-to-six–day-old mosquitoes, respectively.

2.8 Assessing the Flight Ability of *Aedes albopictus* Strains From Different Origins

To assess whether different strains of *Ae. albopictus* exhibit different flight ability scores using the 40-tube FTD (version 1.1), three strains from different origins, Italy (Rimini), Spain (Valencia), and China (Guangzhou), were evaluated. The flight test was repeated twice with three pseudoreplicates per repetition per strain with three-to-four–day-old males. Male mosquitoes were randomly selected from each of the six pseudoreplicates per strain (Rimini: 82; Valencia: 84; and Guangzhou: 79 mosquitoes in total), and their right wings were dissected and measured as a proxy for strain adult size (Nasci, 1990).

2.9 Effects of Fewer Internal Flight Tubes on Escape Rate

To assess whether a reduction of the number of individual internal tubes within the flight test inner tube could affect the performance of the FTD (version 1.1), a FTD was made containing eight individual internal tubes fitted in an inner tube with a diameter of 4 cm (version 2.0). The eight-tube FTD was also tested with both mosquito species. In addition, the effects of cold stress (4°C chilling for 2 hours) and high-dose irradiation (100 and 150 Gy for *Ae. albopictus* and *Ae. aegypti*, respectively) (see **Section 2.1**.) on the sensitivity of the new FTD (version 2.0) were evaluated.

2.9.1 Data Analysis

The escape rate in each experiment was analyzed using a generalized binomial linear mixed-effects model fit by maximum likelihood (Laplace approximation) with a logit link, with the escape rate (proportion of flyers) defined as the dependent variable [whereby escaped (success or flyers) and nonescaped (failure or nonflyers) were weighted with the "cbind ()" function] and replicates as a random effect, considering inferences needed to be done independently of their levels in our specific experimental design (Chaves, 2010).

The fan speed (two levels: normal and high), the test duration (four levels: 30, 60, 90, and 120 min), the adult density per flight test (four levels: 25, 50, 75, and 100 male mosquitoes) were successfully considered as fixed effects in separate models.

To determine the effects of tube color (pink/transparent) and the presence/absence of lure and fan on the escape rates, the tube color, the lure, and fan (coded as "1" for presence and "0" for absence) were considered as fixed effects for each mosquito species/strain. In addition, to assess the effect of a combination of factors defined as flight test treatment (four levels: fan only, lure only, no fan and no lure, and fan and lure for each tube color) on escape rate, treatment was considered as a fixed effect.

Mosquito age (seven and 12 levels for *Ae. aegypti* and *Ae. albopictus*, respectively) was considered as a fixed effect to analyze the effects of age on male flight ability. In addition, four levels (two to three, three to four, four to five, and five to six days) were considered to analyze the effects of age in *Ae. aegypti* (Singapore strain).

Male *Ae. albopictus* mosquito strain (three levels: Rimini, Valencia, and Guangzhou) was considered as a fixed effect to compare flight ability based on origin.

The number of individual internal flight tubes (two levels: 40-tube FTD and eight-tube FTD) and treatments (three levels:

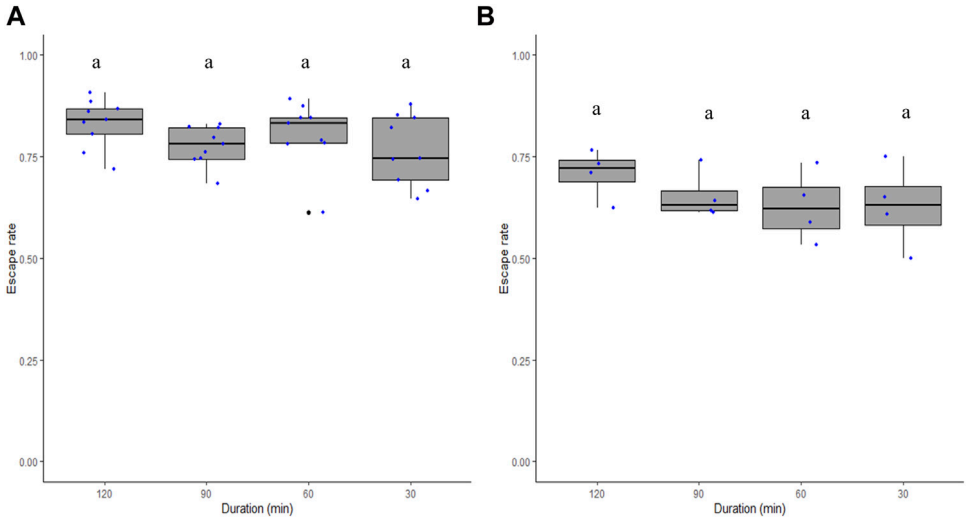

FIGURE 3 | Male *Aedes aegypti* **(A)** and *Aedes albopictus* **(B)** escape rates in response to test duration. Four time periods of 30, 60, 90, and 120 min were tested. Different letters denote significant differences between time periods.

chilled, irradiated, and control) were considered as fixed effects to analyze the effect of FTD type and cold and high-dose irradiation stress conditions on adult flight ability.

For validation, the full models were checked for overdispersion (using Bolker's function) (Bolker, 2018) and for normality and homogeneity of variances on the residuals (Kéry and Hatfield, 2003). When overdispersion in model fit (glmer function) was detected, an individual level random variable was created and added to the model (Harrison, 2015). However, when overdispersion was detected in model fit (glm function), an analysis was performed using quasibinomial errors. The stepwise removal of terms followed by likelihood ratio tests (LRTs) or based on the lowest value of Akaike's Information Criterion (AICc) was used for model simplification. The minimal adequate model retained only factors that significantly reduced explanatory power ($p < 0.05$) when removed (Crawley, 2012). Differences between the levels of significant fixed factors were analyzed using post hoc Tukey's tests (glht function in package multcomp) (Bretz et al., 2016). The significant interactions were analyzed using the emmeans function (in package emmeans) (Lenth, 2020). All statistical analyses were performed using R version 4.0.3 (https://cran.r-project.org) using RStudio (RStudio, Inc. Boston, MA, United States, 2016). All significant differences are based on $p < 0.05$.

3 RESULTS

3.1 Effects of Fan Speed or Airflow on Escape Rate

The fan speed ("normal" 3000 rpm vs. "high" 6000 rpm) had no effect on mosquitoes' escape rate through the FTD in both *Ae. aegypti* [high: 0.76 (0.71–0.81, 95%CI), normal: 0.70 (0.65–0.75, 95%CI), $\chi^2 = 1.17$, df = 1, $p = 0.28$] and *Ae. albopictus* [high: 0.90 (0.87–0.93, 95%CI), normal: 0.91 (0.88–0.94, 95%CI), $\chi^2 = 0.2$, df = 1, $p = 0.65$].

3.2 Effects of Test Duration on Flight Ability

The duration of the flight ability test did not significantly differ between 30, 60, 90, and 120 min in both *Ae. aegypti* ($\chi^2 = 5.35$, df = 3, $p = 0.14$; **Figure 3A**) and *Ae. albopictus* ($\chi^2 = 3.57$, df = 3, $p = 0.31$; **Figure 3B**).

3.3 Effects of Male Density on Escape Rate

The densities of male mosquitoes within the FTD between 25 and 100 did not impact escape rates in *Ae. aegypti* ($\chi^2 = 6.3$, df = 3, $p = 0.09$; **Figure 4A**) but had a significant impact in *Ae. albopictus* ($\chi^2 = 26.2$, df = 3, $p = 0.0001$; **Figure 4B**). The Tukey test shows that a density of 25 male *Ae. albopictus* led to a greater mean escape rate than that of the densities 75 and 100 ($p < 0.05$).

3.4 Effects of Internal Tube Color and Addition of Lure and Fan on Escape Rate of *Aedes aegypti* and *Aedes albopictus*

Greater number of male *Ae. aegypti,* the Brazilian strain, and *Ae. albopictus*, the Italian strain, escaped from the transparent tube more than those that escaped the pink-colored internal tube ($p < 0.001$, **Table 1**). There was a greater impact of the fan on the escape rate of *Ae. aegypti*, Brazilian strain ($p = 0.03$, **Table 1**), and *Ae. aegypti*, Singaporean strain ($p < 0.001$, **Table 1**). The presence of a lure did not enhance the escape rate of both *Ae. aegypti* strains ($p > 0.05$, **Table 1**), but there was a significant interaction between fan and lure on the escape rate of *Ae. albopictus*, Italian strain ($p = 0.01$, **Table 1**).

When considering the treatment of the FTD (i.e., with/without lure, with/without fan, and with/without lure and fan), a significant impact was found on the escape rate of *Ae. aegypti,* Brazilian strain ($\chi^2 = 14.8$, df = 7, $p = 0.03$; **Figure 5A**), *Ae.*

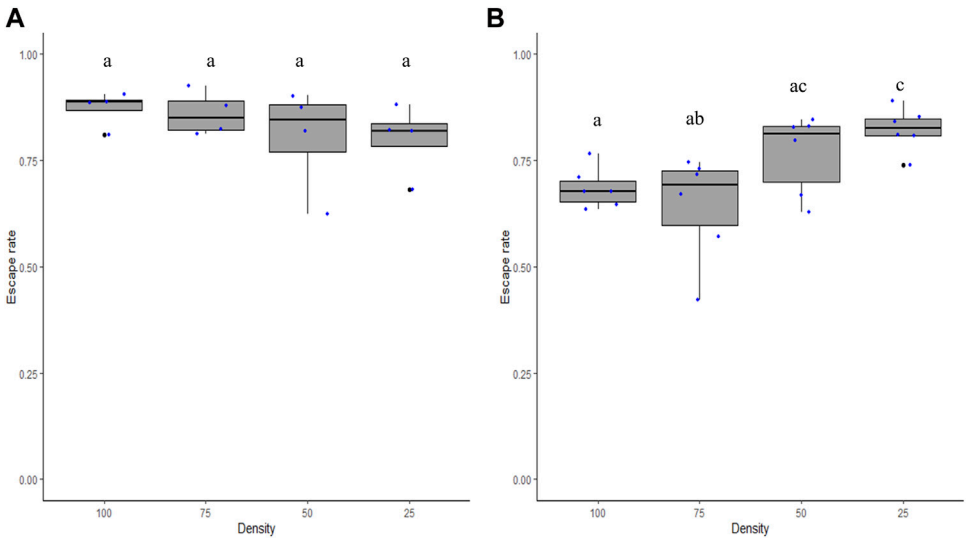

FIGURE 4 | Escape rates according to male density for *Aedes aegypti* **(A)** and *Aedes albopictus* **(B)**. Numbers (25, 50, 75, and 100) stand for the adult male density loaded in each flight test device. Different letters denote significant differences between densities.

TABLE 1 | Fixed effects of the internal tube color and addition of lure and fan (or none) on the escape rates of *Aedes aegypti* (Brazilian strain), *Aedes albopictus* (Italy, Rimini strain), and *Aedes aegypti* (Singaporean strain) males.

Species/strain	Factors	Estimate	Std. error	z value	Pr(>|z|)
Aedes aegypti, Brazil	(Intercept)	0.5765	0.155	3.719	0.0002***
	Transparent	0.342	0.1169	2.925	0.00344**
	Fan1	0.2673	0.1244	2.149	0.03162*
	Lure1	0.2085	0.1283	1.624	0.10427
	(Intercept)	–0.4517	0.1612	–2.803	0.00507 **
	Transparent	1.1402	0.123	9.271	<2e-16 ***
Aedes albopictus, Italy	Fan1	–0.1326	0.1777	–0.746	0.45575
	Lure1	–0.5045	0.2922	–1.726	0.08429
	Fan1 × lure1	0.8285	0.3279	2.527	0.01151 *
	(Intercept)	0.2998	0.11897	2.52	0.01174 *
Aedes aegypti, Singapore	Fan1	0.52074	0.17265	3.016	0.00256 **
	Fure1	0.01417	0.16834	0.084	0.93292

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*
The number "1" following fan and lure stands for their presence as compared to the absence of fan and lure (coded as "0"). The effect of the internal tube color "transparent" was compared to the pink-colored tube.

albopictus, Italian strain (χ^2 = 48.5, df = 7, $p < 0.001$, **Figure 5B**) and *Ae. aegypti,* Singaporean strain (χ^2 = 29.04, df = 3, $p < 0.001$; **Figure 5C**). Furthermore, a higher number of mosquitoes escaped from the reference FTD treatment where a transparent tube, a working fan, and lure were simultaneously used (**Figure 5**). There was a similar number of escapes from the pink-colored FTD in *Ae. aegypti*, Brazilian strain (**Figure 5A**), and *Ae. albopictus*, Italian strain (**Figure 5B**) regardless of the treatment. On the other hand, the difference in the escape rates between the pink-colored tube and the transparent tube tends to be greater in *Ae. albopictus* (**Figure 5B**) than that in *Ae. aegypti* (**Figure 5A**). Although more escapes were recorded from the FTD with fan and lure, they were not significantly different from those from the FTD with a working fan (**Figure 5**). The FTD baited with a lure or without lure and fan displayed lower escape rates than those of the reference FTD and the FTD with a working fan (**Figure 5C**).

3.5 Effects of Male Adult Age on Flight Ability

Age had a significant effect on male flight ability in both *Ae. aegypti* (Brazil strain) (χ^2 = 62.7, df = 6, $p < 0.001$; **Figure 6A**) and *Ae. albopictus* (χ^2 = 1684.4, df = 11, $p < 0.001$; **Figure 6B**). The pairwise comparison of means showed that two-to-three–day-old male *Ae. aegypti* (reference age in Culbert et al., 2018) had higher flight ability than those younger than 2 days ($p < 0.001$) but lower flight ability than males older than 3 days ($p < 0.001$). Similarly, two-to-three–day-old and three-to-four–day-old male *Ae. albopictus* had significantly higher flight ability than those

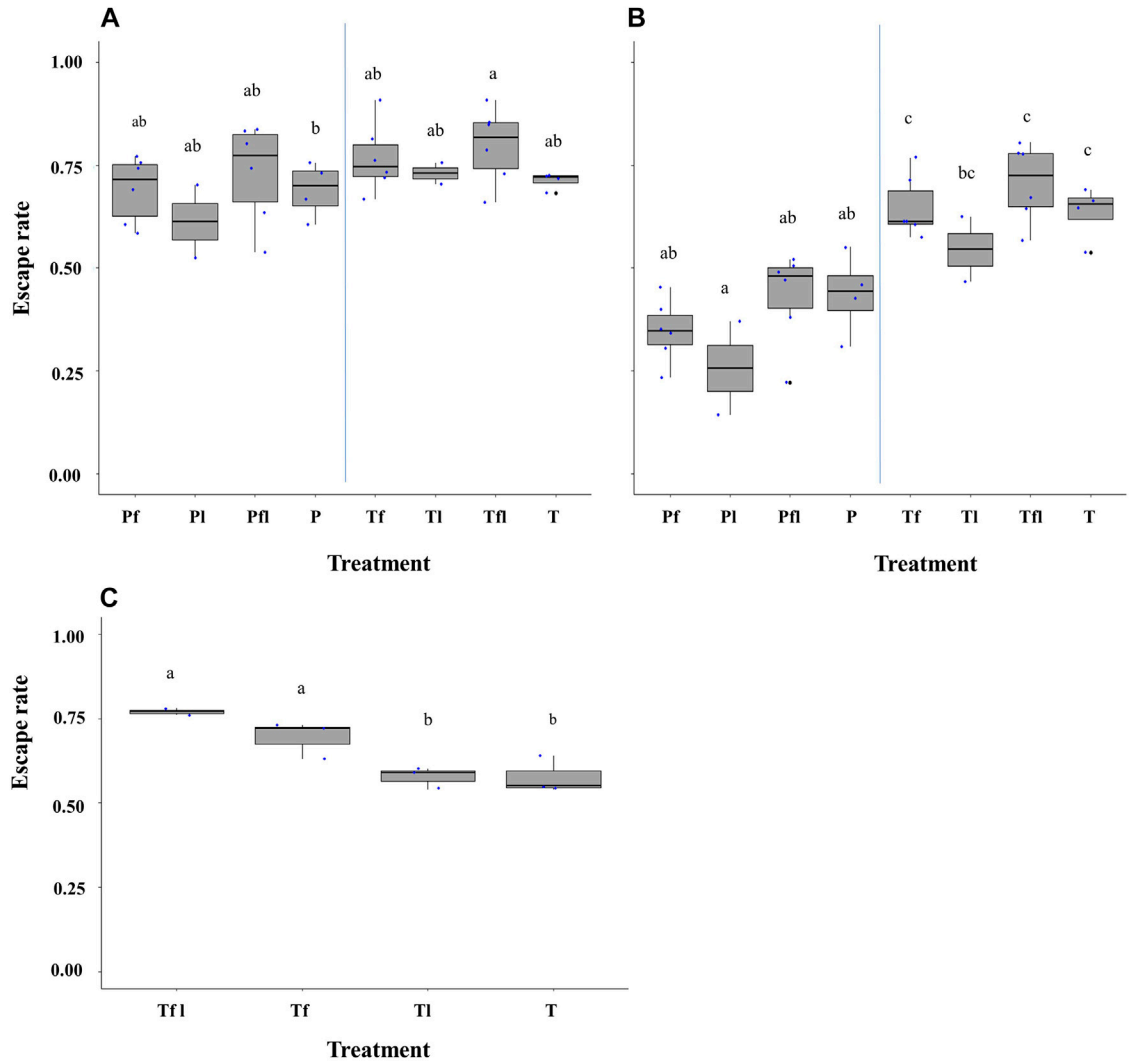

FIGURE 5 | Comparison of escape rates according to the flight tube color and treatment (pink, transparent tubes, with/without lure, with/without fan, and with/without lure and fan) in *Aedes aegypti* (Brazilian strain) **(A)** and *Aedes albopictus* **(B)** and *Aedes aegypti* (Singaporean strain) **(C)**. Pink (P) and transparent (T) stand for flight test device (FTD) tube color (i.e., tube ends with pink silicon or transparent silicon). fl = fan and lure; f = fan only; l = lure only; P/T = pink or transparent without fan and lure. Black bars indicate the median; the upper and lower limits of each box indicate the interquartile range. Each dot represents a value of the observed escape rate per replicate. Different letters denote significant differences between treatments.

younger than 2 days ($p < 0.001$) but lower than males older than 4 days ($p < 0.001$). Flight ability of male *Ae. aegypti* and *Ae. albopictus* declined after the age of five to six and seven to eight days, respectively, leading to a significant decrease in 16-17–days-old male *Ae. albopictus* of ($p < 0.001$) as compared to seven-to-eight–day-old males. Similarly, age had a stronger impact on flight ability in male *Ae. aegypti* (Singapore strain) ($\chi^2 = 62.5$, df = 3, $p < 0.001$; **Figure 6C**).

3.6 Assessing the Flight Ability of *Aedes albopictus* Strains From Different Origins

Similar escape rates of about 74 ± 20 (±95% CI) were observed when different strains of *Aedes albopictus* from different origins (Rimini in Italy; Valencia in Spain; Guangzhou in China) were tested using the 40-tube FTD ($\chi^2 = 0.68$, df = 2, $p = 0.70$) (**Figure 7**).

Wing length of male mosquitoes varied significantly between *Ae. albopictus* strains from different origins ($\chi^2 = 57.03$, df = 2, $p < 0.001$, **Figure 7**). *Aedes albopictus* (Guangzhou strain) exhibited a higher body size as compared to Valencia and Rimini strains ($p < 0.01$), whereas the other two strains have similar sizes ($p = 0.77$, **Supplementary Figure S1**).

3.7 Effects of Fewer Internal Flight Tubes on Escape Rate

When the new FTD with eight individual tubes (version 2.0) was compared to the 40-tube FTD (version 1.1), similar flight capacity was observed both in *Ae. aegypti* ($\chi^2 = 1.26$, df = 1, $p = 0.26$;

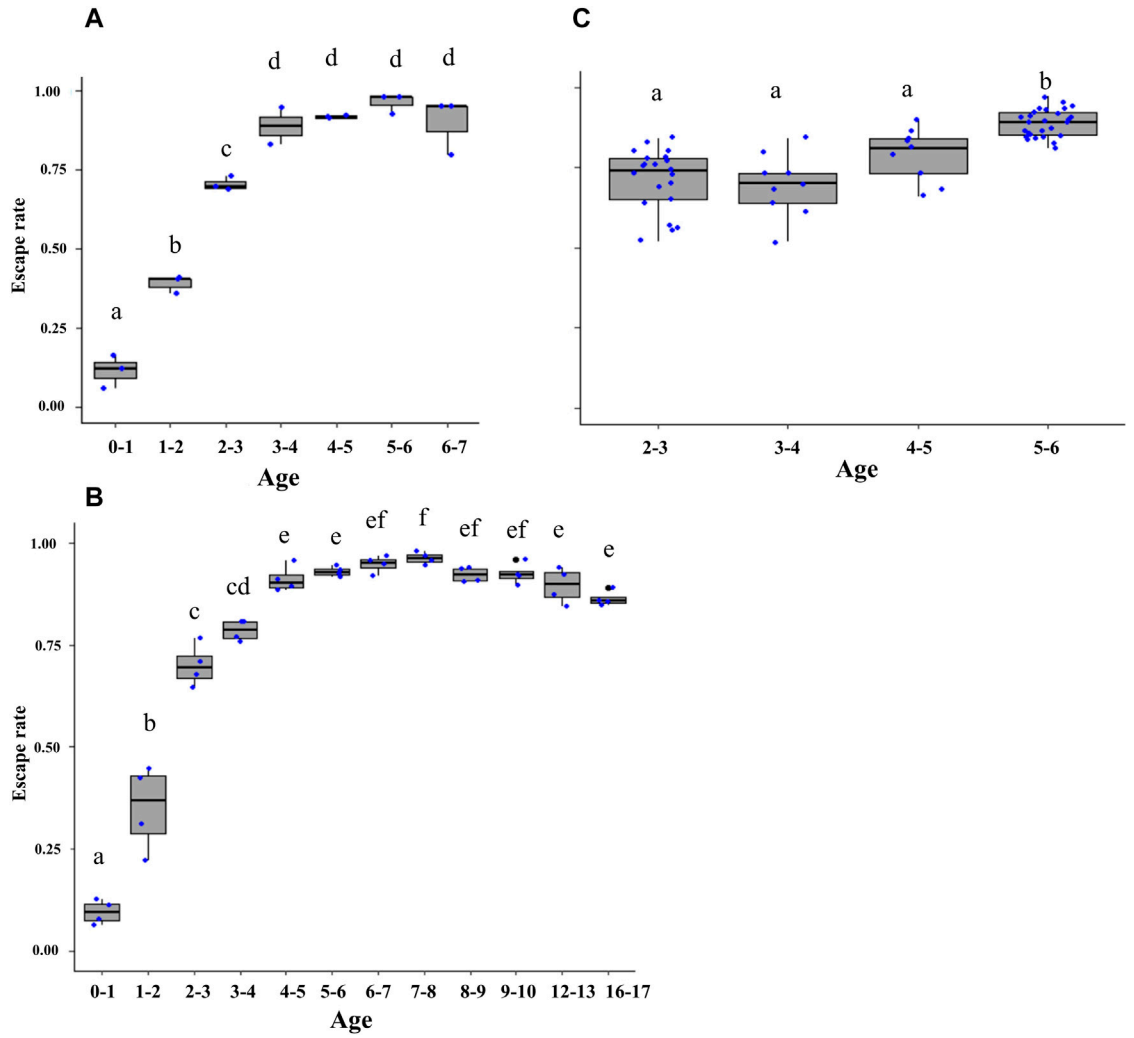

FIGURE 6 | Escape rates across adult male *Aedes aegypti* (Brazilian strain) **(A)**, *Aedes aegypti* (Singaporean strain) **(B)**, and *Aedes albopictus* **(C)** age groups. Age ranged from below one to eight days old **(A)**; from below 1 day to 17 days **(B)**; from two-to-six–day-old mosquitoes **(C)**. Black bars indicate the median; the upper and lower limits of each box indicate the interquartile range. Each dot represents a value of the observed escape rate per replicate. Different letters denote significant differences between age groups.

Figure 8A) and *Ae. albopictus* (χ^2 = 2.86, df = 1, p = 0.09; **Figure 8B**).

The eight-tube FTD (version 2.0) was as sensitive to stress conditions such as cold temperature and high irradiation doses in both *Aedes* mosquito species as the 40-tube FTD (version 1.1) (*Ae. aegypti*: χ^2 = 23.44, df = 2, $p < 0.001$; n = 6, **Figure 8C**; *Ae. albopictus*: χ^2 = 22.38, df = 2, $p < 0.001$, n = 6, **Figure 8D**).

4 DISCUSSION

Our findings demonstrated that the flight tube treatment, including the use of BG-Lure, the use of transparent tubes, and the fan, for a period of 2 h provided consistent and reproducible results for a specified mosquito age group, thus allowing optimal use of the FTD. In addition, a cheaper FTD with only eight tubes was further assessed as a better value QC tool.

It is known that environmental factors, including visual or chemical cues detected by mosquitoes, can affect their behavior (Pitts et al., 2013). The FAO/IAEA reference QC FTD operates with a fan and BG-Lure. The question was whether it could run without either of these without impacting the observed escape rates of mosquitoes from the device. Various airflow rates through fan speeds set at 3000 or 6000 rpm had no effect on the mosquitoes' ability to fly through the FTD, suggesting that this airflow falls within the range for standard operation of the FTD. The presence of a fan (i.e., fan only) showed an increase in mosquito escape rate in *Ae. aegypti* (Brazilian and Singaporean strains). This effect may simply be due to the airflow or the noise of the fan, which was measured at 20.6 dB. Fans are widely used to

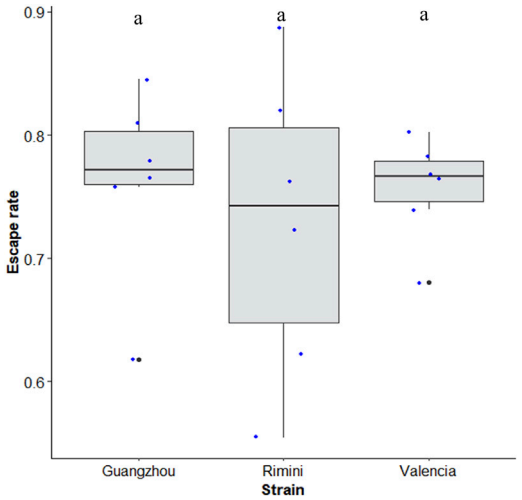

FIGURE 7 | Escape rate of *Aedes albopictus* strains from different origins (Rimini, Italy; Valencia, Spain; and Guangzhou, China). Black bars indicate the median; the upper and lower limits of each box indicate the interquartile range. Each dot represents a value of the observed escape rate per replicate. Different letters denote significant differences between strains.

trap mosquitoes. Depending on the speed, fans produce a characteristic noise that could enhance trap attraction and efficiency (Swan et al., 2021). Dou et al. (2021) assessed the effects of incidental sound stimuli on the flight behavior of free-flying male vs. female *Ae. aegypti* and *Anopheles gambiae* mosquitoes and showed a relative increase in flight speed in response to the stimulus. Conversely, the FTDs baited with or without BG-Lure did not enhance the capacity of males to escape as compared to both the reference FTD and the FTD with a working fan but did reduce variability. This could be since all FTD treatments were run together in the same room and thus, we cannot rule out an additional effect (or interaction) of the lure. Similar results were observed when neither lure nor fan were provided. BG-Lures are blends of mosquito attractants consisting of lactic acid, ammonia, and caproic acid. These components are found on human skin (Kröckel et al., 2006) and are used to mimic human odor. To standardize the use of the FTD, BG-Lure was used to saturate mosquito receptors and reduce their sensitivity to the operators' odor. Potential synergetic effects of the combination of lure and fan led to better escape rates in both *Ae. aegypti* and *Ae. albopictus* mosquito species in our study. Hapairai et al. (2013) showed a significant number of male *Ae. aegypti* collected in French Polynesia using odor-baited BG-Sentinel Traps, while several studies reported the use of BG-Lures in traps to catch male *Aedes* mosquitoes (Pombi et al., 2014; Amos et al., 2020; Visser et al., 2020), Staunton et al. (2021) found during a field study that BG-Lures did not significantly change catch rates and may have even repelled male *Ae. aegypti* and *Ae. albopictus*. A more recent study demonstrated that conflicting results for male *Ae. aegypti* mosquitoes' attraction to humans could be linked to the experimental setting size and assay design for mosquito behavioral research (Amos et al., 2020).

Notably, greater escape rates were observed in *Ae. aegypti* than those in the *Ae. albopictus* mosquito species in our study, meaning that flight behavior may differ between species, highlighting the importance of control groups. Female *Ae. albopictus* were found to be weaker flyers than female *Ae. aegypti* (Briegel et al., 2001). Male *Ae. aegypti* may be more

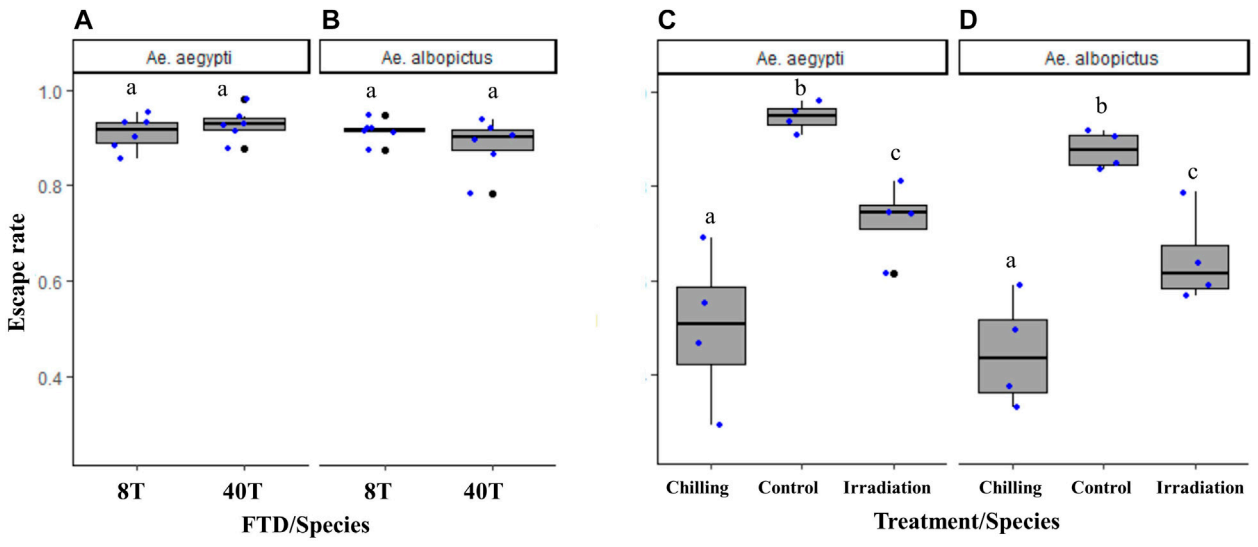

FIGURE 8 | Escape rates of male *Aedes aegypti* **(A)** and *Aedes albopictus* **(B)** in eight-tube FTD ("8T": version 2.0) versus 40-tube FTD ("40T": version 1.1) and effects of cold stress ("chilled") and high-dose irradiation (150 Gy, GammaCell and 100 Gy, X-ray, Raycell) on male *Aedes aegypti* **(C)** and *Aedes albopictus* **(D)** flight ability, respectively, using the eight-tube FTD. Black bars indicate the median; the upper and lower limits of each box indicate the interquartile range. Each dot represents a value of the observed escape rate per replicate. Different letters denote significant differences between FTDs and treatments.

responsive to the device and to different stimuli, including BG-Lure and fan (or their absence), and may tolerate better-shadowed FTDs (**Supplementary Material S16**). These findings highlight the need to avoid any shadows or dark resting sites around the FTD as this may impact flight behavior and cause misleading results regarding mosquito quality based on the escape scores. Shade and vegetation were found to be important determinants of male *Ae. albopictus* catch rate success using BGS traps (Crepeau et al., 2013). In addition, when a black cloth of 8 cm in diameter was placed on top of the pink-colored FTD as an attractant against BG-Lure and fan, it did not improve the observed escape rate in both *Aedes* mosquito species (**Supplementary Material S16**). This also shows that although the color of the fan is black, it did not induce better escape rates.

A quick mosquito flight ability tool is preferable for routine use. Finding a minimum response time for the FTD is important. We found that although no difference in overall escapees for each time period (30, 60, 90, and 120 min) was observed, less variation between replicates was observed for 120 min. Indeed, once mosquitoes are blown into the FTD, they fly upwards through one of the individual internal tubes into the large containment box. However, a proportion of males could be seen resting on top of the inner tube. We cannot rule out that some mosquitoes may return into the individual tubes and so a minimum predetermined duration might be needed for consistent output. Dor et al. (2020) proposed time periods between one and 5 hours to be investigated using their device (80 × 2 cm). In any case, a 2 h time period would be acceptable for a rapid assessment of short-range flight activity as an indicator for their overall quality and performance once released into the field site. However, for this, determining a proxy of male mating capacity based on flight ability would be key. It has been shown that a flight ability score after 2 h could be predictive of survival and insemination rates with over 80% of the inertia (Culbert et al., 2018).

Recent studies on flight assays recommended using two-to-three–day-old adult male *Aedes* mosquitoes for QC (Culbert et al., 2018; Dor et al., 2020). However, it is known that age-related changes in mosquitoes' flight muscles may occur (Johnson and Rowley, 1972) and this may impact flight ability (Rowley and Graham, 1968). Therefore, assessing the flight performance of males of different age groups could help guide the predicted peak age for flight potential for field releases. In our study, although different strains of *Ae. aegypti* from Brazil (maintained at the IPCL) and from Singapore were maintained in different conditions, including larval diet, they both exhibited a significant increase in ability to escape from the FTD with increasing age. This highlights the need for each facility to set up its own QC reference baseline escape rates. We have shown here that mosquito flight ability reference values should be based on a specific age range. Oliva et al. (2012) observed that male *Ae. albopictus* mosquitoes (La Reunion strain) sexual maturation was completed within 13–20 h postemergence and some males were able to inseminate females when 15 h old. However, one-day-old males were less competitive than five-day-old ones in laboratory conditions. Rowley and Graham (1968) indicated that the limiting factor in flight ability appears to be the extent of the glycogen reserves in young mosquitoes, but older mosquitoes are unable to utilize or mobilize glycogen in flight. Increased flight ability with age was also previously demonstrated in insects, including *Drosophila funebris* and *D. melanogaster* (Williams et al., 1943; Wigglesworth, 1949). The response to the FTD could be related to an interaction between age and attractants. This phenomenon was also observed when 10-to-15–day-old mosquitoes were more responsive to CO_2 and human skin odor than younger (three-to-five days old) adults in both *Ae. albopictus* and *Culex quinquefasciatus* (Drago et al., 2021). Sexually immature *Ae. aegypti* males (under 24 h old) exhibit flying but not swarming behavior, according to Cabrera and Jaffe (2007). They also described that sexually mature males (above 24 h of age) initiate a small swarm and secrete an aggregation pheromone, which stimulates and attracts more conspecific males to the swarm. A flight mill assay did not show a difference in mean total flight capability (distance, duration, and velocity) in male *Culex pipiens pallens* (L.) in contrast to their female counterparts (Cui et al., 2013). We also observed that after the age of five to six and seven to eight days, flight ability declined with age in *Ae. aegypti and Ae. albopictus* mosquitoes, respectively. Aging is known as a factor that causes wing damage in house flies (Wehmann et al., 2022), which might impact flight ability.

The FTD has shown its capacity to measure the performance of male *Ae. albopictus* mosquitoes from different geographical origins but maintained in the same conditions. This shows that the device can be widely used in SIT-based control programs against these vectors. Recently, the FTD was used to assess the flight ability of long-term mass-reared *Ae. albopictus* and *Ae. aegypti* mosquito populations (Mamai et al., 2019; Somda et al., 2019; Li et al., 2021). Although there was a significant difference in adult body size between strains, no difference in escape rates was recorded, suggesting that a slight variation in size between strains reared in similar conditions might not limit the use of the FTD. Nevertheless, a recent study has shown that *An. arabiensis*, which are larger than *Aedes* mosquitoes, exhibited fewer escapes from the device. Consequently, a wider diameter of the individual internal tubes (from eight to 10 mm) was proposed for that species (Culbert et al., 2020).

Given the growing number of mosquito SIT programs (Bouyer et al., 2020), a number of IAEA member states are interested in a standardized easy to use and cheap QC method. One of the general challenges of the FTD is its cost. Indeed, the 40 individual tubes of the FTD comprise the majority of the costs of the device in itself. Reducing the number of tubes to eight led to a three-to fivefold reduction in price. A simpler short-flight range device could be designed and created with the aim of further reducing the overall cost while maintaining a 2 h time period for the routine QC assays. Alternative materials to lower the production cost of the flight device could also be investigated in developing countries. Modified gray-colored PVC single-flight tubes with different diameters and heights were tested for male *Ae. aegypti* escape rates after 24 h (Dor et al., 2020). A QC tool that measures mosquitoes' capacity to fly should be sensitive enough to distinguish factors including high-dose irradiation (Parker et al., 2021) and cold stress (Culbert

et al., 2019), as these factors are known to impact male mosquito quality. As such, the observed escape rates using the eight-tube FTD were consistent with similar studies performed with the 40-tube FTD (Culbert et al., 2018, 2020), and thus, we propose this cost-effective version as an alternative.

5 CONCLUSION

This study demonstrated that several factors may influence the measurement of flight performance using the FAO/IAEA reference FTD. The device should be used with a transparent middle flight tube to assess the quality of mass-reared *Ae. aegypti* and *Ae. albopictus* males. The test should be completed with the addition of BG-Lure and fan for a period of 2 hours for a specified mosquito age group. All experiments should be performed in similar conditions of daylight. As this factor may differ between laboratories and settings, it may be worth assessing the effects of different light, temperature, humidity conditions, and the time of the day on the ability of the device to show consistent data. Therefore, each laboratory should meet the minimum environmental conditions to set its own QC figures. Further studies may be required to assess whether the current FTD might allow estimating male mosquito competitiveness.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: HM, GS, RA, and JB; methodology, HM, DL, WM, NB, TW, MB, OB, CM, SK, CC, and JB; software, GS and RA; validation: HM, GS, RA, and JB; formal analysis: HM, DL, and JB; investigation: HM and JB; resources: JB; data curation: HM, DL, and JB; writing—original draft preparation, HM; writing—review and editing: HM, DL, WM, HY, CC, CT, and JB; visualization: HM; supervision: HM, CT, and JB; project administration JB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.876675/full#supplementary-material

Supplementary Figure S1 | Mean wing lengths of *Aedes albopictus* strains from different origins. Strains originated from Rimini, Italy; Valencia, Spain; and Guangzhou, China. Black bars indicate the median; the upper and lower limits of each box indicate the interquartile range. Each dot represents a value of measured wing length.

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Field Performance Assessment of Irradiated *Aedes albopictus* Males Through Mark–Release–Recapture Trials With Multiple Release Points

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Mark–release–recapture (MRR) trials have been conducted in Northern Italy to evaluate the capacity of radio-substerilized *Aedes albopictus* males to survive, disperse, and engage in mating in the field. Two MRR sessions with the human landing collection method (HLC) were conducted with the simultaneous release of irradiated males marked with four different pigment colors. The survival and dispersal rates seem to be influenced more by environmental factors such as barriers, shading, and vegetation rather than weather parameters. In this study, we confirmed a positive linear relationship between the sterile adult male's daily survival rate and the relative humidity previously reported in similar experimental conditions and a different dispersal capacity of the released *A. albopictus* males in low- (NDVI index <0.4) and high (NDVI index >0.4)-vegetated areas. Consistent with previous studies, *A. albopictus* males have their maximal dispersion in the first days after release, while in the following days the males become more stationary. The similar field performances obtained with marked and unmarked radio-sterilized and untreated *A. albopictus* males on similar environments confirm the negligible effects of irradiation and marking procedures on the quality of the males released. The similar sterile to wild (S/W) male ratio measured in high- and low-vegetation areas in the release sites indicates a similar distribution pattern for the wild and the released sterile males. According to the MRR data collected, the Lincoln index estimated different *A. albopictus* mean population densities in the study areas equal to 7,000 and 3,000 male/ha, respectively.

Keywords: sterile insect technique (SIT), dispersal, survival rate (S), normalized difference vegetation index (NDVI), sterile to wild male ratio (S/W)

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INTRODUCTION

The control of urban mosquitoes such as *Aedes aegypti* and *Aedes albopictus* is still an unresolved worldwide problem, as clearly demonstrated by the official number of cases due to the diseases they transmit, such as dengue, Zika, chikungunya, and yellow fever (WHO 2017). The currently applied mosquito control strategies are not achieving the expected results, and the rise of resistance detected in many regions against several key insecticides is alarming and shadowing on the future capacity to

fight mosquito vectors (Ranson et al., 2010; Vontas et al., 2012; Grigoraki et al., 2015). Therefore, new effective tools or strategies to be integrated into the existing ones are under development, including genetic manipulation of vector species, *Wolbachia*-based technologies, autodissemination, and the sterile insect technique (SIT) (Flores and O'Neill 2018; Achee et al., 2019).

The SIT is a species-specific and environment-friendly method of pest control using sequential inundative releases of radio-sterilized males to reduce the reproduction capacity of the target population (FAO 2005). The use of radiation to produce dominant lethal mutations to generate sterile or sub-sterile adult insects has a long history of success (CJEU, 2018; Dyck et al., 2021), has a remarkable lack of resistance (Alphey et al., 2010; Bull 2015), and does not involve the release of insects modified through transgenic engineering processes.

In the past years, a considerable development of SIT application against mosquitoes has been implemented against *A. albopictus* in Italy, and pilot field trials have been conducted in several urban localities to test the performances of sterile males in real condition (Bellini et al., 2013a; Bellini et al., 2021).

In order to integrate the SIT into operational area-wide mosquito management programs, it is essential to verify if mass production, sterilization, and release procedures adopted can negatively affect the quality of the males imposing critical costs. Irradiated sterile males were found to be equivalent to fertile wild competitor under laboratory and semi-field conditions (Bellini et al., 2013b; Madakacherry et al., 2014; Damiens et al., 2016), while the field estimation of the competitiveness index of radio-sterilized *A. albopictus* males under field conditions resulted in a range of 0.03–0.38 (Bellini et al., 2021). One of the most used parameters to measure the effect of the sterile male releases on population suppression is the mating competitiveness value (Fried, 1971) which directly depend on the sterile to male ratio obtained in the field (Bellini et al., 2021). The field evaluation of the sterile to wild male ratio (S/W) provides important indications to modulate the dose and frequency of releases to avoid prolonged period of insufficient presence of sterile males in the field (Hendrichs et al., 2005). The present study was conducted as a collaboration between CAA and the Department of Technical Cooperation of the International Atomic Energy Agency (TC-IAEA) to examine the effectiveness of *A. albopictus* radio-sterilized males to disperse, survive, and effectively compete for mating. The activities described report on the analysis of the survival and dispersal capacities of males during an integrated Aedes vector control program with an SIT component using mark-release-recapture trials with multiple release points.

MATERIALS AND METHODS

Mosquito rearing and sterilization procedures. The mosquito strain used in these trials was started from field materials collected in urban areas of Rimini, Emilia-Romagna (Italy) and maintained for many generations (RN strain F68–F72), under standard laboratory conditions (28 ± 1°C, 80 ± 5% RH, L:D 14:10 h) within the mass rearing pilot module of the Sanitary Entomology and Zoology EZS Department, CAA "G. Nicoli" (Crevalcore, Italy). The rearing methods and conditions used were the same as those described in Balestrino et al. (2017). *Aedes albopictus* male pupae were sorted using metal sieves with square holes of 1,400 μm size, at 24–30 h after the beginning of pupation (Medici et al., 2011), and aged for additional 24 h before irradiation treatment (pupal age at irradiation 24–48 h). Irradiation was performed at the Medical Physics Department of the St. Anna Hospital (Cona, Ferrara, Italy), with a dose of 35 Gy using an IBL 437 irradiator (CIS Bio International, France) equipped with a Cs-137 linear source with a central dose rate of 1.70 ± 3.5% Gy/min (Balestrino et al., 2010). The routine dosimetry and the dose distribution inside the basket are routinely checked using GAFCHROMIC EBT3 dosimetry films (International Specialty Products, Wayne, NJ). After irradiation, the males were transferred back to the laboratory and then placed for emergence inside dedicated cardboard boxes (12 × 12 × 18 cm) with continuous access to cotton pads soaked in a 10% sucrose solution. The radiation dose of 35 Gy was selected as the most effective radiation dose capable to induce in *A. albopictus* a residual fertility of about 1% while maintaining effective flight capacity and quality parameters (Balestrino et al., 2017). In *A. albopictus*, the residual fertility observed at 35 Gy do not affect the mating competitiveness and do not reduce the effectiveness of the technique (Bellini et al., 2013a; Bellini et al., 2021).

Study area. The field trials were conducted in three suburban localities close to the CAA facility: Caselle (44.789718 N, 11.171939 E), Guisa Pepoli (44.702212 N, 11.167757 E), and Bolognina (44.763999 N, 11.146243 E) situated in the municipality of Crevalcore, Bologna province, Northern Italy (**Figure 1**). The villages selected have similar median human population density of about 30 inhabitants per ha and size of 14, 7, and 7 ha, respectively. Each locality is surrounded by rural areas and included usually two-storied houses, separated by narrow lanes, with many private and some public gardens. Larval treatment of permanent breeding sites and removal or inactivation of occasional breeding sites were conducted on monthly basis from May to September 2019 in the three villages in public and private areas (door-to-door campaign) without the application of adulticides treatments (Canali et al., 2017; Donati et al., 2020).

Weather and environmental parameters. Weather parameters such as air temperature, relative humidity, wind (speed and direction), and rainfall were recorded throughout the course of the study by a weather station situated in Sant' Agata Bolognese (Dext3r data; Regional Agency for Environmental Protection Emilia-Romagna Region, ARPAE) a few kilometers from the three study localities. The Normalized Difference Vegetation Index (NDVI) has been used to characterize the study areas for vegetation cover. The NDVI is an indicator of the greenness of the biomes, and two NDVI threshold values able to identify the vegetation types in an urban area were defined; from 0.4 to 1 indicates the medium-high vegetation like trees in urban areas, while a value lower than 0.4 represents low vegetation or non-vegetation. NDVI was calculated from Landsat eight images (https://ers.cr.usgs.gov/) with 30 m resolution, and QGIS 3.16 was used to extract data values on HLC georeferenced points.

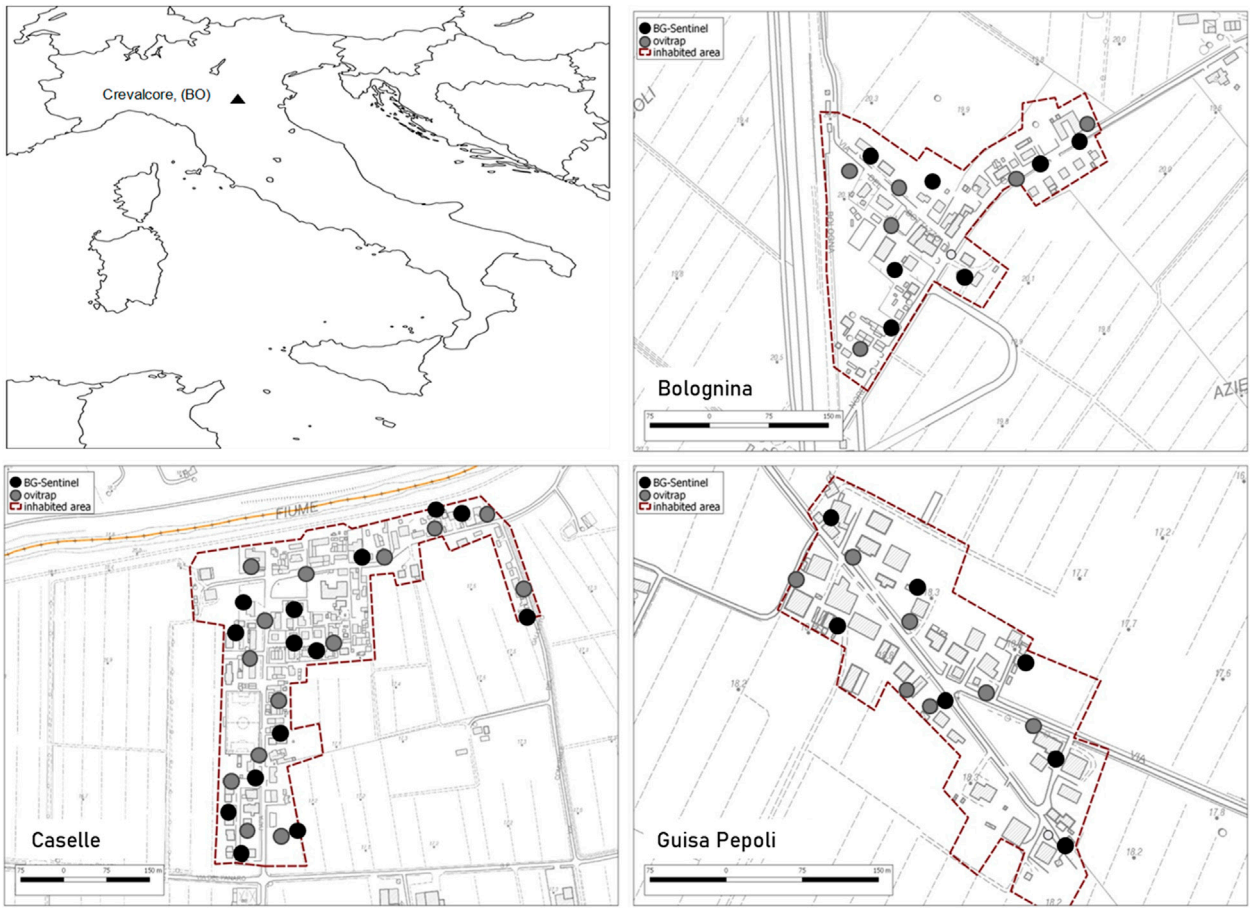

FIGURE 1 | Study areas in Crevalcore, Bologna, Emilia-Romagna, Italy, with indication of the georeferenced monitoring stations (BG-sentinel and ovitraps) in each study localities.

Monitoring system. In all localities**,** a monitoring system at a density of two monitoring stations per hectare was implemented a week before the first male release. Each monitoring station was composed by one BG-sentinel trap baited with standard BG-lure (BG-sentinel 2, Biogents, Regensburg, Germany) and one ovitrap (CAA14GR; Carrieri et al., 2017). Ovitrap consists of 1.4 L black plastic container holding 800 ml of dechlorinated water and a strip of masonite (15 × 2.5 cm) as egg deposition substrate. BG-sentinel traps were activated in the early afternoon at day 7 from the release and operated for 24 h, while ovitraps were placed on the release day, operated continuously, and checked at day 7 from release (**Figure 1**). The mosquito population dynamics was monitored in the release and control areas by counting and hatching the eggs collected in the ovitraps monitoring system. The eggs were counted under a stereomicroscope, embryonated for 7 days, and hatched using a standard procedure to assess their fertility rate in the release areas in comparison with the control area (Bellini et al., 2013a).

MRR trials. To estimate the survival and the dispersal of released sterile males and to measure the sterile to wild male ratio (S/W), two MRR studies were undertaken on July 06–13 and August 03–10, 2019 in the localities of Caselle and Guisa Pepoli. Sterile marked males were released in the MRR areas only (Caselle and Guisa Pepoli), while the recapture sessions were performed in the release and in the control localities (Bolognina). The sterile adult males released in the MRR areas (age 36–48 h) were equally divided and marked while held in the release cardboard boxes using a manual insufflator just before the release, using fluorescent powders of four different colors (Zuper Paint Fluorescent; pink, violet, green, and yellow dust) at a dose of 0.3 g per 1,000 adult (FAO/IAEA 2020). Marked sterile males were ground released by subsequentially opening the cardboard boxes at the four selected release stations employing a different color for each station (**Table1**; **Figure 2**). Distance between release stations was in the range 80–130 m. The release stations selected were usually exposed to the Sun and the releases were performed in the late morning. Mortality of male adults was checked by counting the dead and the not escaped adults observed in the cardboard boxes after 30 min from opening. For each release, a sample of about 300 pupae was withdrawn to check the consistency of the residual presence of females. In each locality, 24 sampling stations distributed to cover an area of about 200 m radius from each release point were

TABLE 1 | Main descriptive data of the MRR trials from rearing to release (Release). NP, number of pupae processed; SA, separation accuracy (sexing method); NP_M, number of pupae male; ER, emergence rate; NA_M, number of adult male; MR, mortality rate of the marked male at release; NA_M_R, number of adult male released; NA_M_R/ha, number of adult male released per hectar (ha) used in the two localities of Caselle (14 ha area) and Guisa Pepoli (7 ha area) during the first and second MRR trial. In HLC is reported the overall number of wild (W_M male and W_F female) and sterile marked adults (Y_M, yellow male; V_M, violet male; G_M, green male; and P_M, pink male) recaptured during the human landing recapture sessions in the different MRR trials. Sterile males were released in Caselle and Guisa Pepoli, while the recapture sessions were performed in all localities. The sterile to wild ratio (S/W) and the sex ratio (SR) calculated in the experimental areas are also reported. In ovitrap, the mean (M ± SD) number of eggs (NE), hatching rate (HR), and the relative Fried male competitiveness index (F_{INDEX}) calculated for each release localities are reported (See also **Supplementary Materials S1–S4**).

Parameter	Date	Area	MRR	NP	SA	NP_M	ER	NA_M	MR	NA_M_R	NA_M_R/ha
Release	06/07/18	Caselle	1	15,000	0.986	14,811	0.910	13,485	0.053	12,768	912
	06/07/18	Guisa	1	7,000	0.986	6,916	0.925	6,397	0.050	6,079	869
	03/08/18	Caselle	2	7,000	0.981	6,867	0.985	6,766	0.052	6,417	458
	03/08/18	Guisa	2	19,000	0.981	18,639	0.976	18,192	0.043	17,404	2,486

Parameter	Date	Area	MRR	W_M	Y_M	V_M	G_M	P_M	W_F	S/W	SR
HLC	07_13/07/18	Caselle	1	756	98	49	42	59	490	0.33	1.54
	07_13/07/18	Guisa	1	357	32	9	83	71	351	0.55	1.02
	07_13/07/18	Bolognina	1	740	-	-	-	-	798	-	0.93
	04_10/08/18	Caselle	2	1,013	9	12	26	13	542	0.06	1.87
	04_10/08/18	Guisa	2	441	116	72	86	121	271	0.90	1.63
	04_10/08/18	Bolognina	2	1,028	—	—	—	—	783	—	1.31

Parameter	Date	Area	MRR	N	NE (M ± SD)		HR (M ± SD)		F_{INDEX}	
Ovitrap	13/07/18	Caselle	1	14	264.6	±157.8	0.834	±0.103	**NS**	0.25
	13/07/18	Guisa	1	7	206.9	±139.0	0.791	±0.166	**NS**	0.26
	13/07/18	Bolognina	1	7	348.6	±178.8	0.904	±0.044		
	10/08/18	Caselle	2	14	409.9	±179.9	0.902	±0.045	**NS**	0.49
	10/08/18	Guisa	2	7	179.3	±112.7	0.671	±0.135	***	0.43
	10/08/18	Bolognina	2	7	534.7	±225.7	0.928	±0.028		

randomly selected in each locality and assigned daily to two operators. In total, 12 operators were rotated daily to avoid potential collection bias. The human landing collection (HLC) sessions were conducted daily starting from the first day after release for seven consecutive days, from 5:00 p.m. to 7:00 p.m. Adult males and females of *A. albopictus* approaching the operator were collected using a manual battery-operated aspirator for 5 min in each sampling station. Each operator was provided with a large black plastic bag filled with polystyrene pieces (30 × 40 × 50 cm; LxWxH) to standardize its visual attraction toward mosquito males and was also provided with a container to transport the adult samples collected. The adult mosquitoes collected were stored overnight at −20°C and screened for identification of species, sex, and marking color the following day under a stereomicroscope using an UV light source. The coordinates of release and recapture stations were entered into an open-source Geographical Information System (QGIS 3.16), to calculate the distance and direction (angles) between each release and recapture site and create thematic maps.

STATISTICAL ANALYSIS

Monitoring system. Linear regression analyses were run to evaluate the relationship between the weekly number of eggs collected in ovitraps and the adults collected with HLC during MRR trials as well as between the weekly number of eggs collected in ovitraps and the adult collected in BG-sentinel traps in the study areas. The relationship between the presence of sterile and wild adult mosquito collected in HLC and the vegetation cover (NDVI Index) was investigated in Guisa Pepoli and Caselle during the second MRR trials. QGIS was used to create an inverse distance weighted (IDW) interpolation raster for marked males in the second MRR session in Guisa Pepoli where we collected a data set sufficient to support statistical evidence. IDW interpolation method was applied to predict the MDT and the S/W values over the entire study area using the measured values surrounding the prediction locations. The measurement of the sterile to wild males' ratio (S/W) was estimated by assessing the mean ratio between marked (sterile) and unmarked (wild) captured males performed the human landing collections (HLC). IDW assumes that each measured point has a local influence on the prediction location that diminishes with distance.

MRR trials. The sterile male dispersal pattern was summarized by the mean distance traveled (MDT), maximum distance traveled (MAX), and flight range (FR) for each locality. Dispersal distance of *A. albopictus* males (MDT) was measured by drawing annuli 50 m apart around the release sites and applying a correction factor to account for unequal

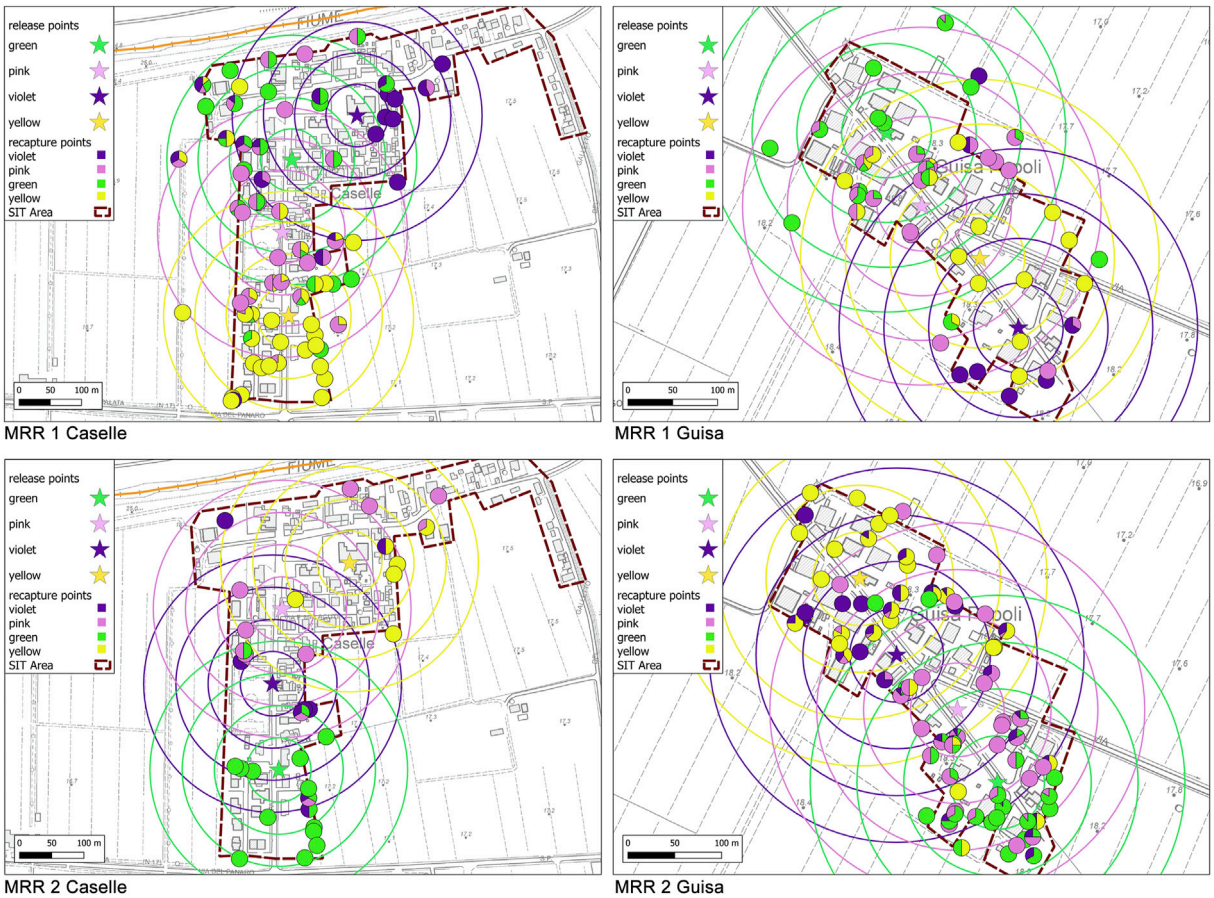

FIGURE 2 | Maps of the recapture points (HLC–circles) located into concentric annuli of 50 m up to the maximum of 200 m radius around the release stations (stars) defined during the different MRR trials in Caselle and Guisa Pepoli. The HLC circles are colored according to the percentage of recapture of males of different colors collected in each station.

trap densities (MDT) as previously described (Le Goff et al., 2019; **Supplementary Materials S1–S4**). Linear regression analyses were performed to evaluate the relationship between the daily distance traveled (MDT) by the sterile males and the day after the release. ANOVAs were performed to evaluate differences in MDT among the two study areas and the NDVI index. The FR was estimated through the linear regression of the cumulative estimated recaptures performed within each annulus (*x*-axis) on the log10 (annulus median distance +1). The FR_{50} and FR_{90} indicate the distance that comprehends the maximum flight distance reached by 50% and 90% of the individuals, respectively. These parameters were calculated from the equation of regression as the value of y at 50% and 90% of the largest value of x, respectively. The mean angle of dispersion (MAD) from the release point (a) was calculated for each locality of release. For each mean angle, the length of the mean vector (r) and the angular deviation (s) was calculated to determine the presence of preferential dispersal directions (from 0 non-directional dispersion to 1 unidirectional dispersion). To determine whether the dispersal direction differed significantly from non-directional uniformity, Rayleigh's test was applied (Bellini et al., 2010; **Supplementary Materials S1–S4**). The probability of daily survival (PDS) was estimated by regressing log10 (x +1) of the number of recaptures against the day of recapture where the antilog_{10} of the slope of the regression line is the PDS. Average life expectancy (ALE) was calculated from the PDS as 1/-loge PDS. The linear corrected method was used to estimate the survival rate. The recapture rates Θ and survival S rates were estimated with equations $\Theta = \hat{e}a/(N + \hat{e}a)$ and $S = \hat{e}b/(1\text{-}\Theta)\hat{}1/d$, respectively, where a and b were the regression coefficients of the linear regression of the ln-transformed captures as a function of time; N is the number of individuals released; Θ is the recapture rate; day is the number of days after release; and S is the daily survival rate (Bellini et al., 2010; **Supplementary Materials S1–S4**).

The model that estimates the sterile male survival rate as a function of the daily relative humidity (RH) previously observed with fertile males in similar environment and described by the formula S = 0.021 RH−0.48 (Bellini et al., 2010; Bellini et al., 2021) was compared with the data observed in the MRR field trials using a *t*-test analysis. The one-way ANOVA of the daily survival rate and mean distance traveled data were compared with MRR data

TABLE 2 | Dispersion and survival parameters registered in the two SIT localities of Caselle and Guisa Pepoli in the first and second MRR trials. *MDT*, mean distance traveled; *MAX*, maximum distance traveled; *FR50%* and *FR90%*, maximum flight distance reached by 50% and 90% of the individuals; *MAD*, mean angle of dispersion (r, length of dispersion; s, angular deviation; a, estimated mean angle; Z value, Rayleigh's test); *Lincoln index-corrected P* indicates the population size expressed in number of male/ha; *PDS*, probability of daily survival; *ALE*, average life expectancy (day); *Θ*, the recapture rate; *S*, the survival rate. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Parameter	Locality	MRR	Y_M	V_M	G_M	P_M	ALL_M (M ± SD)
MAX	Caselle	1	395.4	497.1	306.7	336.3	383.9±84.0
m		2	131.7	276.3	193.0	299.7	225.2±77.3
	Guisa	1	174.0	284.8	276.2	231.7	241.7±50.8
		2	320.7	259.3	231.9	219.8	257.9±45.0
MDT	Caselle	1	146.2	189.7	187.0	245.2	192.0±40.7
m		2	111.0	156.9	127.6	219.2	153.7±47.6
	Guisa	1	107.0	188.1	126.4	87.0	127.1±43.7
		2	160.7	172.4	95.3	74.5	125.7±48.1
FR50%	Caselle	1	74.2	126.8	126.4	188.2	128.9±46.6
m		2	68.5	100.2	81.9	156.4	101.8±38.7
	Guisa	1	64.0	128.6	77.5	39.6	77.4±37.5
		2	101.5	113.9	50.6	32.2	74.6±39.3
FR90%	Caselle	1	274.0	313.7	277.9	493.1	339.7±103.8
m		2	121.4	276.6	167.6	345.2	227.7±101.8
	Guisa	1	173.5	312.0	213.5	168.0	216.8±66.7
		2	328.4	300.7	178.3	141.5	237.2±91.2
MAD	Caselle	1	0.4	0.4	0.2	0.1	0.3±0.1
Length of mean vector (r)		2	0.7	0.2	0.3	0.3	0.4±0.2
	Guisa	1	0.4	0.2	0.1	0.6	0.3±0.2
		2	0.2	0.1	0.2	0.3	0.2±0.1
MAD	Caselle	1	64.5	65.2	70.5	76.6	69.2±5.6
Angular deviation (s)		2	48.1	70.6	67.9	69.7	64.1±10.7
	Guisa	1	64.8	70.5	75.4	49.9	65.2±11.1
		2	74.5	75.2	71.4	66.6	71.9±3.9
MAD	Caselle	1	192.6	82.3	258.4	67.0	150.1±91.4
Estimated mean angle (a)		2	111.2	117.8	137.1	192.5	139.6±36.9
	Guisa	1	317.0	171.3	237.0	352.3	269.4±81.3
		2	111.5	52.9	195.9	205.2	141.4±72.5
MAD	Caselle	1	13.15 ***	6.11 **	2.49	0.65	
Z value (Rayleigh's test)		2	3.78 *	0.7	2.32	0.88	
	Guisa	1	4.16 *	0.53	1.48	27.33 ***	
		2	2.81	1.4	4.30 *	13.82 ***	
Lincoln index corr	Caselle	1	52,048	60,329	117,871	88,038	79,572±29,812
Population size		2	228,119	143,754	45,156	172,380	147,352±76,609
	Guisa	1	12,986	70,514	5,801	7,195	24,124±31,083
		2	11,950	10,669	32,642	17,627	18,222±10,078
Lincoln index cor	Caselle	1	3,253	3,771	7,367	5,502	4,973±1863
Male/ha		2	14,257	8,985	2,822	10,774	9,210±4,788
	Guisa	1	1855	10,073	829	1,028	3,446±4,440
		2	1707	1,524	4,663	2,518	2,603±1,440
PDS	Caselle	1	NA	0.748	0.857	0.777	0.794±0.057
		2	0.871	0.779	0.673	0.825	0.787±0.085
	Guisa	1	0.695	0.837	0.553	0.667	0.688±0.117
		2	0.628	0.600	0.892	0.713	0.708±0.131

(Continued on following page)

TABLE 2 | (*Continued*) Dispersion and survival parameters registered in the two SIT localities of Caselle and Guisa Pepoli in the first and second MRR trials. *MDT*, mean distance traveled; *MAX*, maximum distance traveled; *FR50%* and *FR90%*, maximum flight distance reached by 50% and 90% of the individuals; *MAD*, mean angle of dispersion (r, length of dispersion; s, angular deviation; a, estimated mean angle; Z value, Rayleigh's test); *Lincoln index-corrected P* indicates the population size expressed in number of male/ha; *PDS*, probability of daily survival; *ALE*, average life expectancy (day); **Θ**, the recapture rate; *S*, the survival rate. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Parameter	Locality	MRR	Y_M	V_M	G_M	P_M	ALL_M (M ± SD)
ALE	Caselle	1	NA	3,440	6,485	3,971	4,632±1,627
Day		2	7,213	4,004	2,522	5,204	4,736±1,983
	Guisa	1	2,744	5,634	1,690	2,472	3,135±1,725
		2	2,150	1,960	8,747	2,954	3,953±3,225
Recapture rate, Θ	Caselle	1	0.009	0.008	0.004	0.005	0.006±0.002
Per 1 ha released		2	0.003	0.004	0.011	0.004	0.005±0.004
	Guisa	1	0.015	0.002	0.026	0.036	0.020±0.015
		2	0.025	0.033	0.011	0.017	0.022±0.010
Survival rate, S	Caselle	1	0.826	0.733	0.845	0.831	0.809±0.051
		2	0.873	0.783	0.680	0.828	0.791±0.083
	Guisa	1	0.674	0.874	0.568	0.633	0.687±0.132
		2	0.561	0.507	0.731	0.681	0.620±0.104

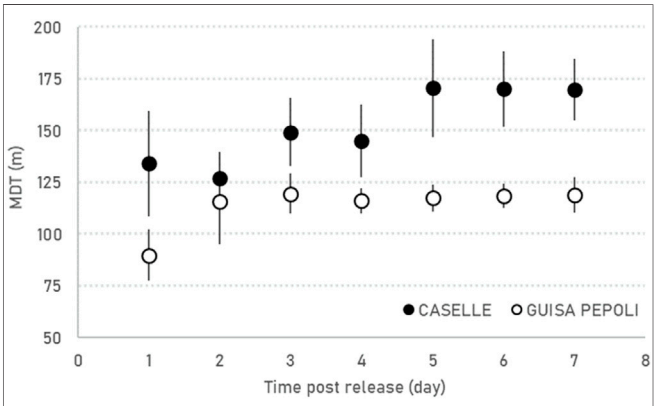

FIGURE 3 | Daily mean (±SE) distance traveled (MDT) by sterile marked males recorded in the MRR trials in Caselle and Guisa Pepoli.

obtained in 2010 with *A. albopictus* fertile males marked with fluorescent pigments or by eliminating *Wolbachia* symbiont (aposymbiotic strain) in suburban localities with similar environmental conditions (Bellini et al., 2010).

The Lincoln index modified for a low recapture rate and compensated for daily survival (P) was used to estimate the wild male population size for the different batches used in each locality at each day after release. The modified Lincoln index is calculated as P = [R*St (n-m+1)]/(m+1), where S is daily survival rate and t is sampling day after release, R is the number of marked males, n is the total number of recaptures of both marked and wild adult males, and m is the number of recaptured marked males (Le Goff et al., 2019; **Supplementary Materials S1–S4**).

The fertility rate of the eggs collected in the release and control sites allow to estimate the sterile male competitiveness index under field conditions using the Fried competitiveness index (Fried, 1971) calculated as F = W/S * [(PW–PS)/(PS–PRS)], where PW is the mean natural fertility in the control site of Bolognina, PS is the fertility rate observed in the release area, and PRS is the residual fertility of the released males. The PRS was close to zero and always below 1% and therefore we not considered this correction in the competitiveness index formula (Bellini et al., 2013a; FAO/IAEA 2020, **Supplementary Materials S1–S4**). All statistical analyses were conducted using STATISTICA 7.0 software package (StatSoft Inc., United States) and are reported in the **Supplementary Materials S1–S4**.

RESULTS

Monitoring system. The weekly mean number of eggs per ovitrap (E) shows a significant linear relationship with the number of wild males (M_{HLC} = 0.011 *E + 0.265; R^2 = 0.83; $F_{1,4}$ = 19.1; p = 0.012) and wild females (F_{HLC} = 0.008 *E + 0.62; R^2 = 0.69, $F_{1,4}$ = 9.0; p = 0.039) observed in the study area by the HLC collections while no correlation was observed between HLC adult collection and BG-sentinel traps catches, either for wild females (R^2 = 0.69; p = 0.13), wild males (R^2 = 0.14; p = 0.79), or released sterile males (R^2 = 0.35; p = 0.65).

MRR trials. In the localities of Caselle and Guisa Pepoli, about 19,000 and 23,500 marked males were released in the two MRR trials (**Table 1**), respectively, with a residual presence of females equal to 1.65% (±0.36%) on the total number of released adults. The mean (±SD) mortality rate observed following the release of sterile marked males was equal to 4.9 (±0.4%) (**Table 1**), which was similar to the mortality rates reported in the literature during MRR trial with fertile *A. albopictus* males marked with fluorescent dust (Bellini et al., 2010; Gouagna et al., 2015). The overall released males in the different MRR trials and the HLC mean recapture data are summarized in **Table 1** and further data are reported in the

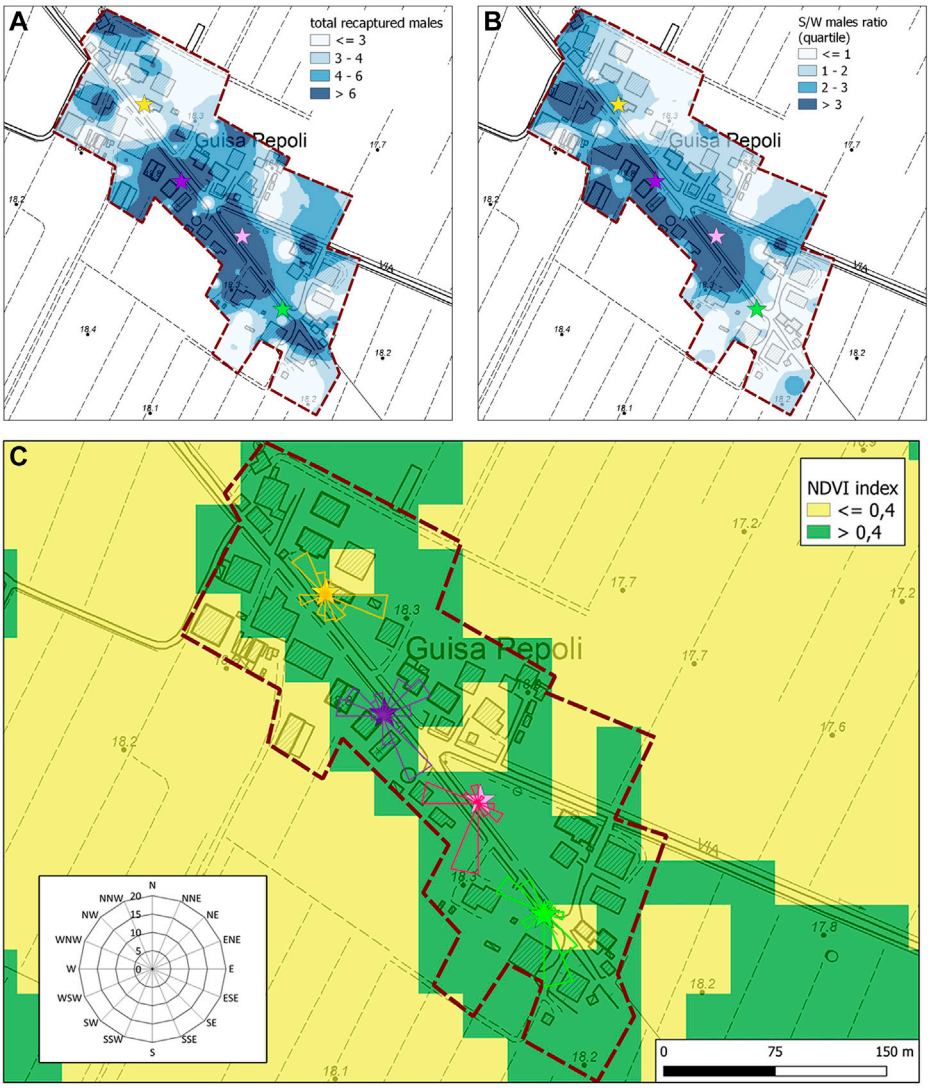

FIGURE 4 | Summary map of **(A)** the total recaptured males and **(B)** S/W ratio of the recaptured males (IDW interpolation rasters) in Guisa Pepoli during the second MRR trial. Colored stars represent the release points. **(C)** Normalized difference vegetation index (NDVI) map and direction dispersion of sterile males in Guisa Pepoli in the second MRR trial. The direction and the dimension of the bands in the rose diagram show the direction and intensity of the sterile male's distribution.

Supplementary Materials S1–S4. The maps of the release and recapture points in the two MRR trials for each locality are shown in **Figure 2**.

The sterile males have similar MDT increase rate over time in the two localities but in Caselle the males showed a significant ($F_{1,14}$ = 4.64; p = 0.049) higher MDT (172.9 ± 45.8 m) in comparison with Guisa Pepoli (MDT 126.4 ± 44.6 m) (**Table 2**; **Figure 3**). The sterile males showed their maximal daily dispersion on day one post-release, while in the following days, the sterile males continue to disperse from the release station with a lower and constant intensity (**Table 2**; **Figure 3**). The mean (±SD) distance traveled (MDT) by the sterile males at the third day after release in Guisa Pepoli was about 95.9% (±2.8%) of their mean dispersal capacity (MDT), while in Caselle the sterile males covered 97.4% (±3.7%) of their overall displacement at the fifth day after release. The overall mean (±SD) distances within which the 50% (FR_{50}) and 90% (FR_{90}) of the released males were re-collected and the maximal distance (MAX) traveled by males in Caselle (FR_{50} = 115.3 ± 42.2 m; FR_{90} = 283.7 ± 112.5 m; MAX = 304.5 ± 113.1 m) are all higher than values measured in Guisa Pepoli (FR_{50} = 76.0 ± 35.7 m; FR_{90} = 227.0 ± 74.8 m; MAX = 249.8 ± 45.2 m) but not significantly different (FR_{50}: p = 0.064; FR_{90}: p = 0.255; MAX: p = 0.224).

We also found some evidence of statistically significant preferential dispersion directions with unidirectional tendency in either the first and second MRR trials in both localities without a specific correlation with any weather or environmental parameters (MAD z values in **Table 2** and **Figure 4A**).

The mean NDVI index in the different HLC recollection sites differs significantly ($F_{1,286}$ = 23.8; $p < 0.0001$) among the two MRR areas (Caselle: 0.59 ± 0.15; Guisa Pepoli: 0.51 ± 0.13). The analysis conducted on the second MRR's data indicate a more uniform distribution of wild and sterile mosquito captures in Caselle with no difference in catches performed in medium–high (>0.4) and low (<0.4) NDVI index for wild female ($F_{1,166}$ = 1.81; p = 0.18), wild males ($F_{1,166}$ = 0.44; p = 0.51), and sterile males ($F_{1,166}$ = 0.00; p = 0.98), respectively. In Guisa Pepoli, the females were uniformly distributed irrespective of the vegetation ($F_{1,166}$ = 0.24; p = 0.63), while the number of wild ($F_{1,166}$ = 7.62; p = 0.01) and sterile males catches ($F_{1,166}$ = 4.55; p = 0.02) were significantly higher in areas with medium–high vegetation index. The sterile male distribution pattern in Guisa Pepoli during the second MRR trial showed a high concentration of males in the central area which is characterized by high NDVI values (**Figures 4A, B**). While the overall mean sterile to wild male ratio (S/W ± SD) in Guisa Pepoli was equal to 1.24 (±2.83), in the central part of the study area the S/W ratio exceed 3. The similar S/W male ratio measured in Caselle ($F_{1,125}$ = 0.52; p = 0.47) and in Guisa Pepoli ($F_{1,103}$ = 0.04; p = 0.84) in areas with different vegetation index, indicates a similar distribution pattern for the wild and the released sterile males (**Figure 4C**).

Results of daily survival probability (PDS) and average life expectancy (ALE) of marked sterile males are presented in **Table 2**. Whatever the color the mean (±SD) values of the ALE were not different ($F_{1,13}$ = 1.09; p = 0.315) in the two localities and varied from 3.54 (±2.43) to 4.69 (±1.69) days in Guisa Pepoli and in Caselle, respectively. The PDS were also similar in the localities tested ($F_{1,13}$ = 3.37; p = 0.09) varying from 0.55 to 0.89 with a mean (±SD) value of 0.70 (±0.12) and 0.79 (±0.07), respectively, for Guisa Pepoli and Caselle. The overall mean (±SD) survival rate measured during the MRR trials in Caselle (80.0 ± 6.4%) was significantly higher ($F_{1,13}$ = 9.80; p = 0.007) than values observed in Guisa Pepoli (65.4 ± 11.5%). The daily survival rate ranged from 68 to 87% and from 51 to 87% in Caselle and Guisa Pepoli, respectively. The overall mean recapture rate achieved in Guisa Pepoli (2.1 ± 1.1%) was significantly higher ($F_{1,14}$ = 12.5; p = 0.003) than the recapture rate obtained in Caselle (0.59 ± 0.3%). The overall cumulative estimation of the mean recapture rates during the MRR trials ranged from 0.26 to 1.08% and from 0.21 to 3.58% in Caselle and Guisa Pepoli, respectively (**Table 2**).

No differences (t-test; p = 0.65) were observed between the daily survival rate values (s) directly observed in the MRR trials (0.76 ± 0.06) and estimated based on the RH values (0.74 ± 0.05) using the model described previously. The sterile *A. albopictus* males have an overall higher ($F_{2,18}$ = 7.49; p = 0.005) mean daily survival rate in comparison with not irradiated *A. albopictus* males (0.46 ± 0.08) marked with higher quantity of fluorescent dust and released in similar environments in previous MRR trials (Bellini et al., 2010). A similar daily survival rate (0.79 ± 0.24) was instead observed in comparison with fertile males marked without the use of fluorescent dust (aposymbiotic strain) (Bellini et al., 2010).

Using the Lincoln index, the overall mean size (±SD) of the population estimated in Caselle (89,632 ± 44,795) and in Guisa Pepoli (44,795 ± 21,622) were different ($F_{1,14}$ = 15.15; p = 0.0016) with an higher mean number of male per ha ($F_{1,14}$ = 5.09; p = 0.041) observed in Caselle (7,091 ± 4,054) in comparison with Guisa Pepoli (3,024 ± 3,089). The mean value (SD) of the daily population size and density estimations for each group of mosquitoes marked with different colors are reported in **Table 2**.

The overall mean (±SD) residual fertility of *A. albopictus* males irradiated at 35 Gy at the pupal stage and measured on caged individuals in laboratory conditions (Balestrino et al., 2010) was equal to 1.00 ± 0.72% (**Supplementary Materials S1–S4**). According to the model developed by Aronna and Dumont (2020), it is necessary to limit the residual fertility of the released males according to the population basic offspring number and anyway below 2% to achieve an effective control of *A. albopictus* populations in a tropical area using the SIT as part of an integrated pest management (IPM) approach. Considering a population abundance similar to that described for the same tropical areas (Erguler et al., 2017), the use of radiation doses between 30 and 40 Gy is confirmed to be the most effective dose to effectively induce sterility into the natural population with an acceptable residual fertility lower than 2% (Balestrino et al., 2010; Bellini et al., 2013a; Bellini et al., 2021). At these doses the mating competitiveness of males and the effectiveness of the SIT technique for the suppression of *A. albopictus* populations has already been validated in the laboratory (Balestrino et al., 2017; Culbert et al., 2018) and in field temperate areas (Bellini et al., 2013a; Bellini et al., 2021). The overall mean (±SD) natural fertility calculated in the untreated control area of Bolognina during the MRR study period was 91.6 ± 3.8, while in the release sites the observed fertility was equal to 73.1 (±4.69) and 86.8 (±8.50), respectively, in Guisa Pepoli and Caselle (**Table 1**). The fertility rate of the eggs collected in Caselle and in Guisa Pepoli during the MRR trials were all lower than the fertility eggs registered in the control area of Bolognina in the same periods. The sterile male release rates achieved in these trials were equal to 912–458 males/ha in Caselle and 868–2,486 in Guisa Pepoli, respectively, in MRR1 and MRR2 (**Table 1**, NA_M_R/ha). However, the fertility rate difference observed in the eggs collected in the field was significant only in Guisa Pepoli during the MRR2 ($F_{1,12}$ = 24.4, p = 0.0003). The competitiveness index was calculated in Caselle and Guisa Pepoli during the first and the second MRR trials (range 0.25–0.49; **Table 1**) even if the only significant value for this parameter was collected in Guisa Pepoli during MRR2 (0.43, **Table 1**).

DISCUSSION

The mass rearing methods applied at the CAA facilities in 2018 allowed a male recovery rate of about 20% of the reared males, with a mean female contamination of 1.65%. These rearing

parameters are currently one of the most limiting factors affecting the development of a cost-effective large-scale implementation of SIT against *A. albopictus*. The females accidentally irradiated and released with males become permanently sterilized (Balestrino et al., 2010) but still maintain their biting activity with a short-term risk of increased arboviral disease transmission. Even if the accidental release of females could not sensibly increase the epidemiological risk of disease transmission and do not seriously affect the SIT effectiveness (Dumont and Yatat-Djeumen, 2022), their presence could affect the political and ethical acceptability of this technique, especially in areas where mosquito-borne diseases are endemic, hence the recommendation to keep it below 1% (WHO/IAEA, 2020). A more effective and consistent sex sorting system is therefore strongly needed to increase the male productivity and the sex separation accuracy, as pointed out recently (Lutrat et al., 2019). In a recent successful trial in China, the authors reported a female contamination of 0.3% for a male recovery of 70% which offers new perspective for future SIT trials (Zheng et al., 2019).

As previously showed (Carrieri et al., 2011; Bellini et al., 2013b), the ovitrap monitoring system as employed in the three study localities can provide a good estimation of the *A. albopictus* wild population density. The authors of this study reported a highly positive correlation between the number of pupae per hectare (PHI) and the weekly mean number of eggs per ovitrap collected in similar field environment in northern Italy. The distribution and availability of competitive artificial containers could modify the female oviposition behavior in ovitraps, thus influencing the relationship between the number of females and the number of eggs in the ovitraps. However, according to the findings described by Carrieri et al. (2011), in *A. albopictus*, the ovipositing females did not seem to be influenced by the egg density in the available breeding sites. This aspect together with the uniform distribution of the most productive larval habitats (catch basins) in Italy allowed the effective use of egg density data from ovitraps to infer the mean number of adults per unit area. A significant positive relationship between ovitrap data and data from HLC was also reported in field trials carried out in the metropolitan area of Rome, Italy (Manica et al., 2017). The authors of this study confirmed the possibility to successfully predict the mean number of adult biting females in HLC based on the mean weekly number of eggs collected in the field. Our results, based on a limited data set, are in line with these results and support the conclusion that the relationship between the number of eggs and the wild adult population can be effectively and significantly established in Italy thanks to the capillary distribution of the available breeding sites, while a more generalized relationship between eggs density in ovitraps and wild adult population cannot be inferred for different environment without specific field investigations. The BG-sentinel traps activated for 24 h once a week as employed in our studies produced data not sufficiently accurate to effectively estimate the adult population dynamics in the study areas. However, daily sampling comparison between BG-sentinel trap baited with BG-Lure and CO2 and HLC method demonstrated similar trapping efficiency and provides similar estimations of the main entomological parameters during MRR trials with *Aedes albopictus* E. Velo, personal communication.

The mean recapture rates obtained in our trials (0.27–1.7%), despite the large variability observed between localities and sessions, may be considered within the range usually found in mark-release-recapture studies (Service 1993; Caputo et al., 2021) and is not different from values observed in *A. albopictus* fertile males marked with fluorescent dust in the same environment with similar recapture density (Bellini et al., 2010).

The recapture rate strongly depends on the dispersal capacity of the target species, on the recapture effort (density of recapture stations) and on the efficacy of recapture method employed (Service 1993).

The radio-sterilized *A. albopictus* males showed an overall MDT and survival rate not different from values registered for this species in different MRR studies with either sterile or fertile individuals (Bellini et al., 2010; Gouagna et al., 2015; Iyaloo et al., 2019). It is interesting to note that the overall MDT value obtained in this study using radio-sterilized *A. albopictus* males marked with fluorescent powders (MDT = 149.6 ± 48.9 m) is not different (p = 0.51) from the MDT obtained with not irradiated *A. albopictus* males (MDT = 124.4 ± 21.2 m) similarly marked with fluorescent dust and released in similar environments (Bellini et al., 2010).

While we cannot exclude that fluorescent dust-based marking procedures may sensibly increase mortality and decrease mobility of marked mosquito (Dickens and Brant, 2014), the similar field performances obtained between sterile and fertile *A. albopictus* males released in similar environmental condition indicate the negligible effects of the irradiation dose (35 Gy) on the quality of the sterile males released.

In this study we confirmed the positive linear relationship between sterile adult male survival rate (s) and relative humidity (RH), as previously reported (Bellini et al., 2010; Bellini et al., 2021). Adult dehydration caused by high temperatures and low RH is likely to be an important factor affecting survival (Costa et al., 2010) and could influence the population size of these insects in the environment (Alto and Juliano 2001).

However, the observed survival and dispersion seems to be influenced more by environmental factors such as barriers, shading and vegetation rather than weather parameters. Among the environmental factors, the vegetation coverage can strongly influence the abundance of *A. albopictus* and can positively affect their dispersal capacity and distribution (Ayllón et al., 2018; Iyaloo et al., 2019). The positive correlation we observed between the NDVI index and the sterile male density confirm the important role of the vegetation coverage in the dispersal of the released *A. albopictus* males. The sterile males need to disperse from the release sites to reach natural courtship and mating arenas where wild males and females are already present. Soon after the release, the sterile males are still more concentrated and active in the proximity of the release sites while not yet dispersed in more

distant areas. Consistent with previous studies (Bellini et al., 2010), in the first day following the release males have their maximal dispersion while in the following days the males move less and with a reduced directionality.

The S/W ratio measured showed a large variability and its variance is higher than the mean value suggesting a cluster distribution of the released sterile males, at least until the second or third day after release. The S/W male ratio measured in these MRR field trials, was also used to determine the mean (±SD) sterile male competitiveness in Guisa Pepoli and Caselle which confirmed the effective mating capacity of the released radio-sterilized males in the field (Bellini et al., 2021). Mass production, manipulation, irradiation and transportation procedures can affect the performance of the sterile adult males in the field. The competitiveness indices calculated during these trials indicate an effective mating behavior of the released sterile males in the target field areas even if they should be interpreted with caution since they were reported for a single week of release and differences in hatch rates between the control and release areas were not all significant. However, in operational SIT release campaigns performed in the same environments a release rate of 896–1,590 males/ha per week was effective to suppress wild populations (Bellini et al., 2013a) with effective field competitiveness (Bellini et al., 2021). The sterile male field competitiveness is directly affected by the spatial distribution of the sterile and wild males in the field and a release method which allows a more homogeneous distribution of the sterile males in the field could probably assist the SIT programs effectiveness. The possibility to release sterile adult mosquitoes males by a drone was already demonstrated for Aedes mosquitos in the field with substantial reduction of the operational release costs (Bouyer et al., 2020). The aerial release systems would also allow males to be released in a closely controlled manner into specific geographic locations difficult to access and according with the local population density.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FB, AP, MC, JB, and RB conceived and designed experiments. FB, AP, MM, and AA performed laboratory work for sterile male production. FB, AP, MM, AA, MC, and RB performed laboratory and field experiments. AA, MC, and JB performed data analysis. JB and RB provided funding and supervised experiments. FB, AP, MC, and RB wrote the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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A Mark-Release-Recapture Study to Estimate Field Performance of Imported Radio-Sterilized Male *Aedes albopictus* in Albania

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The pathogen transmitting *Aedes albopictus* mosquito is spreading rapidly in Europe, putting millions of humans and animals at risk. This species is well-established in Albania since its first detection in 1979. The sterile insect technique (SIT) is increasingly gaining momentum worldwide as a component of area-wide-integrated pest management. However, estimating how the sterile males will perform in the field and the size of target populations is crucial for better decision-making, designing and elaborating appropriate SIT pilot trials, and subsequent large-scale release strategies. A mark-release-recapture (MRR) experiment was carried out in Albania within a highly urbanized area in the city of Tirana. The radio-sterilized adults of *Ae. albopictus* Albania strain males were transported by plane from Centro Agricoltura Ambiente (CAA) mass-production facility (Bologna, Italy), where they were reared. In Albania, sterile males were sugar-fed, marked with fluorescent powder, and released. The aim of this study was to estimate, under field conditions, their dispersal capacity, probability of daily survival and competitiveness, and the size of the target population. In addition, two adult mosquito collection methods were also evaluated: BG-Sentinel traps baited with BG-Lure and CO_2, (BGS) versus human landing catch (HLC). The overall recapture rates did not differ significantly between the two methods (2.36% and 1.57% of the total male

released were recaptured respectively by BGS and HLC), suggesting a similar trapping efficiency under these conditions. Sterile males traveled a mean distance of 93.85 ± 42.58 m and dispersed up to 258 m. Moreover, they were observed living in the field up to 15 days after release with an average life expectancy of 4.26 ± 0.80 days. Whether mosquitoes were marked with green, blue, yellow, or pink, released at 3.00 p.m. or 6.00 p.m., there was no significant difference in the recapture, dispersal, and survival rates in the field. The Fried competitiveness index was estimated at 0.28. This mark-release-recapture study provided important data for better decision-making and planning before moving to pilot SIT trials in Albania. Moreover, it also showed that both BG-traps and HLC were successful in monitoring adult mosquitoes and provided similar estimations of the main entomological parameters needed.

Keywords: mosquitoes, pest, management, survival, dispersal, competitiveness, BG sentinel trap, Sterile Insect Technique

INTRODUCTION

Mosquitoes represent a threat to both human and animal health. They are vectors of various diseases such as malaria, dengue, chikungunya, Japanese encephalitis, West Nile virus, Rift Valley fever, yellow fever, and Zika and lymphatic filariasis (Tolle, 2009; Kampen et al., 2012).

The invasive tiger mosquito *Aedes (Stegomya) albopictus* (Skuse, 1895), native to Southeast Asia, has colonized all continents (Benedict et al., 2007). It was introduced to Europe at the end of the 20th century (Lounibos, 2002; Scholte and Schaffner, 2007; Medlock et al., 2012), and its first occurrence was reported in Albania in 1979 (Adhami and Reiter, 1998). The species is now well-established and present even in tiny isolated villages in high-altitude (>1,200 m) environments (Tisseuil et al., 2018). Although the risk for pathogen transmission related to the autochthonous *Aedes* species is currently considered low, intensified vector control measures are needed to prevent disease outbreaks similar to those that occurred in various countries with unexpected local cases of chikungunya, dengue, and Zika (ECDC, 2019; Vermeulen et al., 2020). Even in the absence of disease transmission, *Ae. albopictus* is a significant nuisance species in urban areas (Kolimenakis et al., 2019).

Since its first report from Albania, it has been recorded in numerous European countries (Medlock et al., 2012; Medlock et al., 2015) and became well-established across Mediterranean countries, and more recently it spread northward to Germany (Becker et al., 2017), North Macedonia (Cvetkovikj et al., 2020) and Moldova (Şuleşco et al., 2021), and Austria (Schoener et al., 2019; Bakran-Lebl et al., 2021).

European health officials are concerned about the risk posed by invasive mosquito species to public health, and the World Health Organization (WHO) office for Europe is suggesting to rapidly develop the required capacities to face the problem (Bellini et al., 2020). Due to the rapid spread of resistance to commonly used insecticides (Hemingway and Ranson, 2000; Vontas et al., 2012; Grigoraki et al., 2017; Pichler et al., 2018), integrated vector management including new methods is now widely accepted, and this strategy is emphasized in the Global Vector Control Response (WHO, 2017). The sterile insect technique (SIT), an insect birth control method, has historically been used to suppress and even eradicate several agricultural and livestock/human pests (Vreysen et al., 2000; Dyck et al., 2021). In response to increasing demand for SIT application from the International Atomic Energy Agency (IAEA)/Food and Agriculture Organization of the United Nations (FAO) Member States, substantial efforts have been invested in the development of the SIT package against mosquitoes including the development of equipment and protocols for mass-rearing, sex-separation, irradiation, handling, packing, transport, release, and quality control (Balestrino et al., 2014a; Balestrino et al., 2014b; Bimbilé-Somda et al., 2019; Culbert et al., 2019, 2020; FAO/IAEA, 2020b; Maiga et al., 2016; Maïga et al., 2017; Maïga et al., 2019; Mamai et al., 2017; Mamai et al., 2019a; Mamai et al., 2019b; Yamada et al., 2019; Zheng et al., 2015). The WHO and IAEA have recently published a joint guidance framework for testing SIT as a vector control tool against *Aedes*-borne diseases (WHO/IAEA, 2020).

Understanding the bio-ecological features of the target population and how laboratory-produced sterile males may perform in the natural environment is crucial. Mark-release-recapture (MRR) studies are particularly useful and have been frequently applied to various insect species to study characteristics of populations related to the ecology, biology, behavior, ability to transmit pathogens, and ultimately their control (Gillies, 1961; Pollock, 1991; Hagler and Jackson, 2001; Silver, 2007; Bellini et al., 2010; Epopa et al., 2017; Benedict et al., 2018; Oliva et al., 2021). Knowledge of the characteristics of sterile males and reliable quantification of wild population density are prerequisites for planning SIT interventions (Bouyer et al., 2020b; Romeis et al., 2020; Oliva et al., 2021). The few MRR studies available for radio-sterilized *Ae. albopictus* males (Iyaloo et al., 2020) and non-radio-sterilized males (Le-Goff et al., 2019) mainly aimed at assessing survival, dispersal, and/or population size analysis. Only four field estimations of competitiveness are currently available (Zheng et al., 2019; Bouyer et al., 2020a; Iyaloo et al., 2020; Bellini et al., 2021). An appropriate method

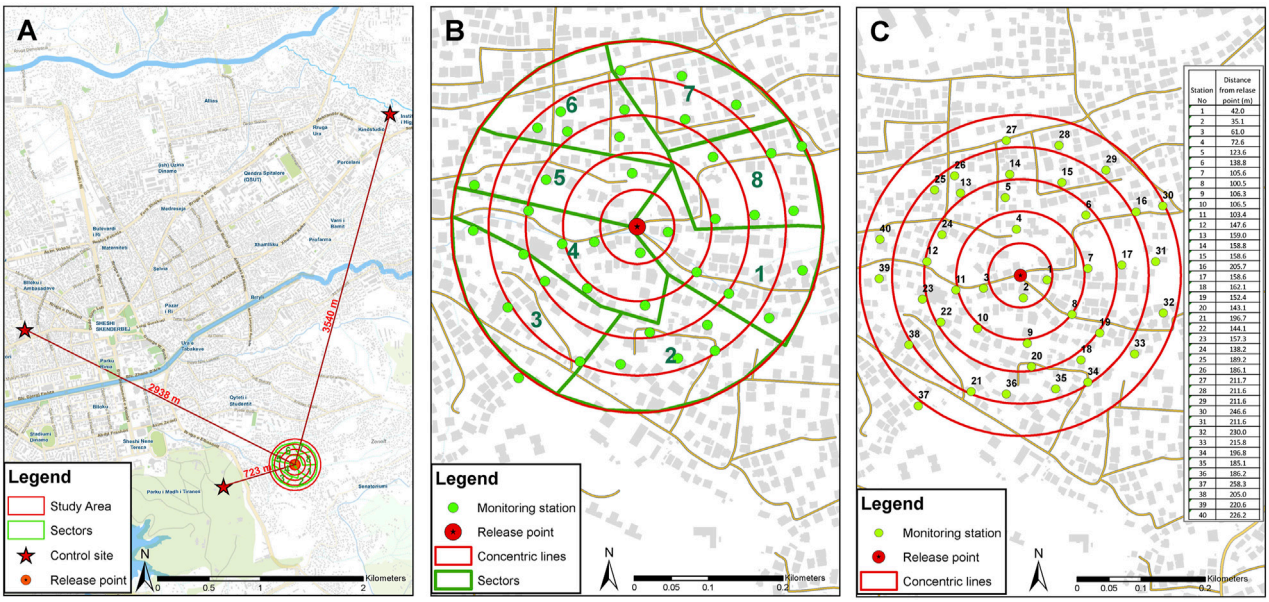

FIGURE1 | Map of mark release recapture setup in Tirana and distribution of 40 trapping stations for mosquito collection (two stations per hectare). **(A)** Location of release and control sites. **(B)** Study area is divided into sectors for simultaneous monitoring by different teams (green lines). The red stars represent the control sites, 700, 3,040, and 3,440 m from the release site. **(C)** Positions and number of trapping stations. Concentric red lines represent five annuli at 50, 100, 150, 200, and 250 m from the release point (red point in the center).

for population monitoring is also necessary to apply SIT. Although the BG-Sentinel trap (BGS) is considered the gold standard method for catching *Aedes* mosquitoes (Williams et al., 2006; Farajollahi et al., 2009; Staunton et al., 2020), some factors including shade, presence of bushes, and potential larval habitats were shown to influence its efficacy (Staunton et al., 2020).

In this study, we investigated the performance of a radio-sterilized local strain of *Ae. albopictus* using MRR. Specifically, parameters that were assessed included 1) recapture rate, 2) probability of daily survival, 3) dispersal capacity, 4) sterile-to-wild male ratio, 5) wild population estimation, and 6) field competitiveness. We also exploited the data to compare the efficiency of two adult mosquito trapping methods to estimate these parameters, namely, the BG-Sentinel 2™ used with BG-Lure® and CO_2 and the human landing catch.

MATERIAL AND METHODS

Study Site

The MRR study was conducted in an urban area of Tirana (41°19′44″N, 19°49′04″E), the capital and the largest city of Albania. The presence and establishment of *Ae. albopictus* in the area have been proven via monitoring activities since 2010. The area is characterized by two-storied houses with many private and some public gardens (**Figure 1**). The field monitoring was conducted by ovitraps following the standard operating procedure by Bellini et al. (2021), with slight modifications (filter paper instead of Masonite paddle as oviposition substrate). The egg monitoring started in May 2017 (week 22) before the sterile male releases, to allow the required field data collection on population dynamics and egg fertility (unpublished data). Fertility of wild eggs was assessed by standardized hatching procedures (see **Supplementary material S1**) during the MRR trials on egg samples collected both in the release area and in separated control areas (700 m West-Southwest, 3040 m Northwest, and 3440 m North from the release site) with comparable land use and cover.

Origin of the Mosquitoes and Rearing Procedures

Mosquitoes released in this MRR experiment were obtained from *Ae. albopictus* eggs collected in Albania (ALB strain) and amplified in the biosafety level 2 (BSL-2) laboratory of the Medical and Veterinary Department of the Environmental and Agriculture Centre "G. Nicoli" at CAA (Bologna, Italy). Research carried out on mosquitoes in confined laboratory conditions do not require a specific permit according to the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Adult mosquitoes were maintained following environmental and rearing conditions described in Balestrino et al. (2014b). The second generation (F2) was sent to Albania for the MRR study.

Sexing and Transportation

Sex separation was carried out according to the standard method of sieving, at 24–30 h from the beginning of pupation (Bellini et al., 2007; Medici et al., 2011). The residual amount of females in

TABLE 1 | Number of released and recaptured radio-sterilized males *Aedes albopictus*.

Color	Release date	Time of release	Initial males delivered and marked	Mortality before release (%)	Males that flew	Number (%) recaptured with BGS	Number (%) recaptured with HLC	Number (%) total recaptured
Green	06/09/2017	3:00 p.m.	16,000	24.04	12,124	222 (1.83)	102 (0.84)	324 (2.67)
Orange	06/09/2017	6:00 p.m.	16,000	27.58	11,558	288 (2.49)	158 (1.37)	446 (3.86)
Yellow	13/09/2017	3:00 p.m.	16,000	22.66	12,344	325 (2.63)	269 (2.18)	594 (4.81)
Pink	13/09/2017	6:00 p.m.	14,000	14.39	11,985	296 (2.47)	227 (1.89)	523 (4.36)
Total			62,000	22.56	48,011	1,131 (2.36)	756 (1.57)	1887 (3.93)

male pupae was 1%. The pupae collected were aliquoted in batches of 2,000 pupae each and transferred into plastic containers of 500 ml capacity filled with 200 ml of water. These containers were used to transport the pupae to the Medical Physics Department of the St. Anna Hospital (Cona, Ferrara, Italy) for irradiation and brought back afterward to the laboratory for packaging and adult emergence. During transportation, the pupae were maintained in thermal insulated plastic containers with changing phase materials (PCM ice gel packs; Blu Ice, Dryce Srl, Milano, Italy) to maintain a temperature of about 15°C.

Irradiation Treatments

Irradiation treatments were performed using an IBL 437 irradiator (CIS Bio International, Bagnols-sur-Ceze, France) equipped with a 50.9 TBq Cs-137 linear source with a central dose rate of 1.8 Gy/min. At the hospital, the different batches of pupae were transferred into separated petri dishes (12 cm diameter) filled with 25–30 ml of water and piled inside a dedicated canister for irradiation (Balestrino et al., 2010). The dose of gamma rays administered to the pupae was equal to 40 Gy.

Packaging and Shipment

At the CAA laboratory, the irradiated pupae inside petri dishes with water (25–30 ml) were placed at the bottom of cardboard boxes ($12cm^3 \times 12cm^3 \times 18\ cm^3$) closed at the top with a mosquito net for emergence and transportation. Cotton pads soaked with 10% sugar solution were provided and secured at the top of each box to assure adult nourishment. Additional separator partitions were added inside each box to provide an overall vertical resting surface area of 1.04 cm^2 per adult. The mosquito boxes were maintained at about 20°C for the first two days after adult emergence, and after removing water from the petri dishes, the boxes were transferred inside a larger polystyrene container with an adequate quantity of ice gel packs to maintain a temperature between 10 and 15°C during transportation to the release site. In order to maintain this temperature range over 24–48 h, we used a 70-L polystyrene container filled with 10 kg of ice gel packs conditioned at 4°C (PCM Blu Ice) and 2 kg at −20°C (PCM Green Ice, Dryce Srl, Milano, Italy). A data logger was also introduced inside each container to register the environmental conditions during transport.

Mosquito Marking Procedure

Within 3 h of reception, the males were sugar-fed and marked with fluorescent dust (RADGLO® JST, Radiant NV, Houthalen, Belgium) (FAO/IAEA, 2020a) applied using a bulb duster to create a dust storm within the transportation cardboard boxes (**Supplementary material S2**). A fixed dose of fluorescent powder equal to 0.6 gr (0.3 gr per 1,000 adult) was used per each cardboard box and manually insufflated to disperse the powder uniformly on mosquitoes just before the release. Culbert et al. (2020) have shown that with the current dose of the fluorescent dust used, there was no effect on the survival of *Aedes* mosquitoes. Fluorescent dust coverage on male body parts was evaluated on a sample of about 300 mosquitoes randomly collected from the different cardboard boxes upon each release.

Mosquito Release and Recapture

The MRR study was undertaken from 4th September to 2nd October 2017. One release of sterile marked males per week was performed using different colors on two consecutive weeks. The mosquito release point was located in the center of the 20-ha study area (**Figure 1**), where 40 sites were selected to sample the mosquito population homogenously (two sampling stations per hectare). The sampling sites were arranged the day before the first males' release in five successive concentric circles placed 50 m apart starting from the central release point (**Figure 1A**). In each sampling station, a pair of traps were set and spaced 10–20 m apart, consisting of a BG-Sentinel 2™ (Biogents, Regensburg, Germany—BGS) baited with dry ice (1 kg/24 h) and BG-Lure™ (Biogents, Regensburg, Germany) and an ovitrap made from a 500-ml black polypropylene cup (Luwasa Interhydro AG, Allmendingen, Switzerland; 11/9 hydroculture pot; 11 cm diameter x 9 cm high) lined with heavy-weight seed germination paper (#76 seed germination paper, Extra Heavy Weight, Anchor Paper Co., St Paul, MN, United States). Ovitrap and BGS placed in the same sampling station were coded with the same station number.

Right after marking (**Supplementary material S2**), the mosquito males were transferred to the center of the study area for release. The dusted males were released as young adults (72–96 h-old) by placing them on the ground and opening the cardboard boxes in a shaded area. The boxes were gently shaken to induce the males to exit. The males that

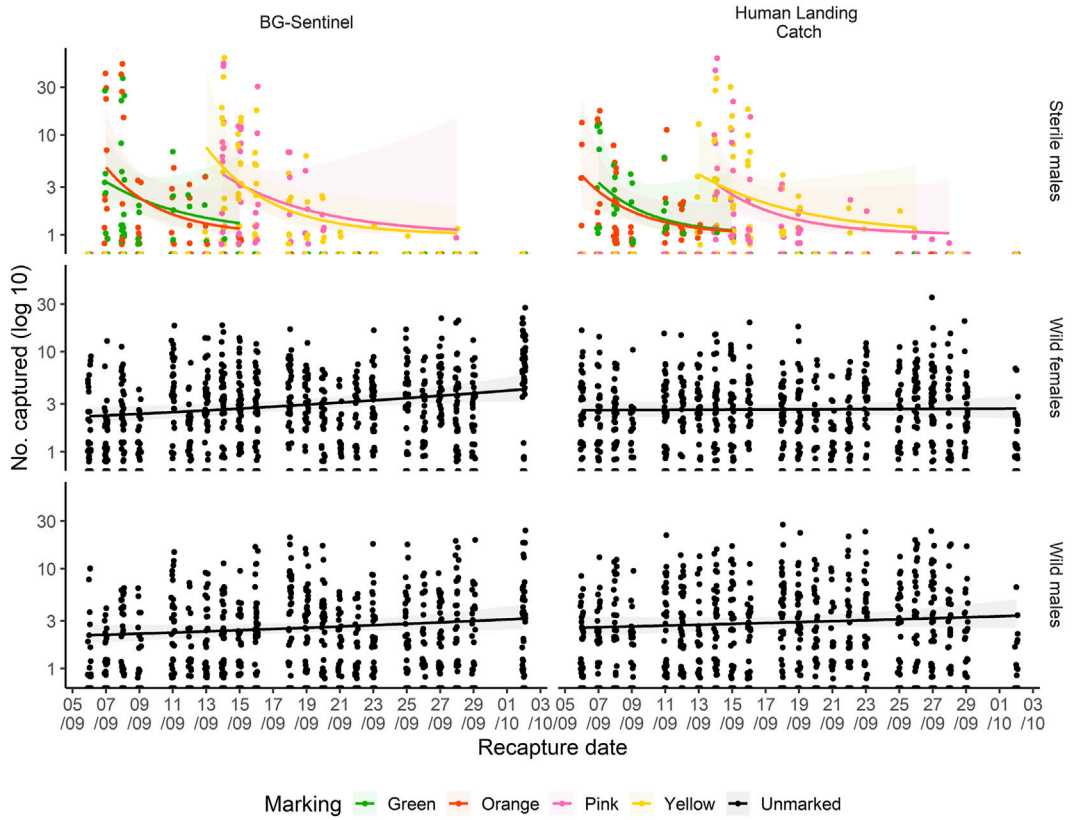

FIGURE 2 | Capture of dusted-marked sterile males, wild male and female (both unmarked) mosquitoes as a function of time (September and October 2017) elapsed since release using BG-Sentinel trap and human landing catch. The regression line shows the trend of increase/decrease.

remained in the cage after 30 min were considered dead. The two releases of *Ae. albopictus* sterile males were in the amount of ca 30,000–32,000 males each. The release dates were September 6 (16,000 orange marked sterile males at 3:00 p.m. plus 16,000 green marked sterile males at 6:00 p.m.) and September 13 (16,000 yellow marked sterile males at 3:00 p.m. plus 14,000 pink marked sterile males at 6:00 p.m.). Eight teams were involved in this study. Teams included expert entomologists who decided the exact position of each trap in the field according to the available environment characteristics, prefered by *Ae. albopictus*. Each team was in charge to place and daily inspect four to six stations. Starting from the first trap positioning and during every visit, each team collected *Ae. albopictus* adult males and females flying around the team members (human landing catch, HLC) by handmade manual battery aspirators (12 V DC, 0.19 A, aspiration 2.5 m/s) (Balestrino et al., 2017) for 15 min in each sampling station. Only one operator per team performed the HLC, and the collected adults were recorded with an identification code referring to the sampling station and the collection date. The collection of samples (HLC, BGS, and ovitrap) from different sites was conducted every day from 3:00 p.m. to 6:00 p.m., except on Sunday. In case of unfavorable weather conditions (rainfalls), the HLC was not conducted. The sequence of stations was randomly rotated daily at each visit to avoid possible collection time bias. Each team was provided with an insulated thermal container to transport dry ice and store and euthanize the adult samples collected (during the daily collection). Collected adult mosquitoes were stored overnight and screened for identification of species and coloration the following day under a stereomicroscope and using a 12-V UV lamp. The use of a UV light source was to facilitate the identification of fluorescent dust on the male mosquito collected.

Egg papers collected from ovitraps were stored in plastic bags (not completely sealed) and transported at ambient temperature. The number of eggs on each paper was counted the following day, and the portion of the paper with eggs was cut out in strips, stored, maturated, and hatched at least 7 days after the collection day according to the procedures in **Supplementary Material S1A**. Each day after collection, the adult field data samples were analyzed, classified, and stored into a Geographical Information System platform to calculate the distances between release and each recapture site. In this trial, a dedicated WebGIS application available at https://invent.al/produkte/index.html under the MRR training link was used to georeference and manage the field data collected. BGS were stopped after two trapping sessions without any collection of marked males, and after this date, only ovitraps were monitored for 4 additional weeks. The activity continued this scheme until October 02, which was 19 days after the last release. Weather parameters were collected from a meteorological

station located in the neighborhood (for rainfall and wind speed) (see **Supplementary Material Figure S1**; **Supplementary Table S1**). In parallel, in three other areas nearby, where no sterile males were released (control areas, see **Figure 1A**), five ovitraps were positioned in each and checked weekly to estimate the natural egg fertility and to measure possible failure to hatch caused by diapause.

Statistical Analysis

Statistical analyses were performed using R Software version 4.1.0 (R Development Core Team 2008; https://www.R-project.org/). A generalized linear model (with Poisson family distribution and log as link function) was used to check for difference in the main parameters between trapping methods, marking colors, and time of release. For the difference in the natural egg fertility between the control sites, we used a binomial generalized linear mixed model fit by maximum likelihood (Laplace Approximation) with egg hatch as the response variable and the ovitrap station as a random effect. The male dispersal has been analyzed by the mean distance traveled (MDT), the maximum distance traveled (MAX), and the flight range (FR). Dispersal distance estimation of *Ae. albopictus* males was facilitated by the homogeneous density of recapture stations in the study area. The FR was estimated through the linear regression of the cumulative estimated recaptures performed in each recapture stations (x-axis) on the log10 (annulus median distance). The FR_{50} and FR_{90} indicate the distance that comprehends the maximum flight distance reached by 50% and 90% of the individuals, respectively. These parameters were calculated from the equation of regression as the value of the y axis at 50% and 90% of the largest value of x, respectively. A random isotropic model in two dimensions has also been fitted to the data to calculate the diffusion coefficient.

The survival rate of sterile males was estimated by the linear corrected method (Harrington et al., 2001; Buonaccorsi et al., 2003) as follows: $\theta = e^{\alpha}/(N+ e^{\alpha})$, $S = e^{b}/(1- \theta)^{1/d}$, where a and b are the regression coefficients of the linear regression of the log-transformed captures as a function of time, N is the number of individuals released, θ is the recapture rate, d is the number of days after release, and s the survival rate. The probability of daily survival (PDS) in the field is estimated by regressing $\log_{10}$ (x +1) of the number of recaptures against the day of recapture where the antilog_{10} of the slope of the regression line is the PDS (Muir and Kay, 1998). Average life expectancy in the field (ALE) is calculated from the PDS as 1/- $\log_e$ PDS.

Based on the sterile males released and recaptured, the wild male population size in the study area was estimated using the modified Lincoln index that corrects for small samples and compensates for daily survival P = [R*S (n − m + 1)]/(m + 1), where R is the number of originally marked males, S is the daily survival rate, n is the total number of recaptures of both marked and wild adult males, and m is the number of recaptured marked males. These data, together with the fertility rate of the eggs (hatched eggs and normally shaped eggs with the presence of embryo are considered fertile) collected in the release and control sites allow us to estimate the sterile male competitiveness index under field conditions using the Fried competitiveness index (Fried, 1971) as follows: F = ((Ha–Ee)/Ee)/R, where Ha = natural fertility in the control site (determined during the MRR feasibility study period) and Ee = observed fertility rate, R = ratio of sterile over wild males. Using data from BGS, a nonparametric bootstrap approach (Efron, 1979) was applied to obtain a confidence interval for the estimate of the Fried index as described in Bouyer et al. (2020a). In brief, the data on fertility and the ratio of sterile males over wild ones were resampled without replacement, and for each set of resampled data, the Fried index was computed (1,000 simulations). Assuming a symmetric distribution, the basic percentile method to get a 95% confidence interval was used.

RESULTS

Environmental Weather Conditions in the Study Area During the Mark-Release-Recapture Experiment

The mean daily temperature in Tirana for the whole 2017 years ranged from −3.46°C to 33.51°C (mean ± se = 17.65 ± 0.40°C). The environmental weather data variation and the average of the main variables over the MRR experimental period (September 2017 and October 2017) are presented in **Supplementary Material Table S1** ; **Supplementary Figure S1**). The mean (daily) temperature during the MRR ranged from 12.46 to 27.08 C (mean ± se = 19.60 ± 0.44°C). The mean daily relative humidity for 2017 ranged from 32.69% to 100% (mean ± se = 60.74% ± 0.66%). For September and October, the mean relative humidity ranged from 47.07% to 84.14% (mean ± se = 64.43 ± 1.03%). No rainfall was recorded during the two days before the first release. However, the day before the second release, there were strong rainfalls followed by minor rainfalls during the day of release. The daily wind speed was constant (**Supplementary Table S1**), ranging from 0.9 to 1.5 m/s.

Recapture of *Aedes albopictus* and Trap Efficiency

Out of the estimated 62,000 color-marked sterile males, a total of 48,011 flew from the release boxes and 1,887 were recaptured throughout the 3-week collection period by both methods, representing 3.93% of the total males released (**Table 1**). Out of these recaptured sterile males, 59.94% were obtained from BGS (2.36% of the total males released), while the remainder 40.06% (1.57% of the total males released) from HLC. Considering the entire period of collection, there were no statistical difference in the recapture rates of sterile males between the trapping methods BGS and HLC (GLM, df = 1, F = 3.53, p = 0.06) and dust marking colors (GLM, df = 3, F = 0.406, p = 0.7490). However, total captures of wild males and females were much higher with BGS than with HLC (GLM, df = 1, F = 11.23, p = 0.0008 and df = 1, F = 54.12, p = 2.13e-13 for males and females, respectively). Moreover, the time of release (3: 00 p.m. or 6:00 p.m.) did not impact the recapture rate of the released sterile males (GLM, df = 1, F = 0.836, p = 0.361).

Figure 2 shows variation in the recapture of sterile males as well as of wild mosquitoes with both methods as a function of

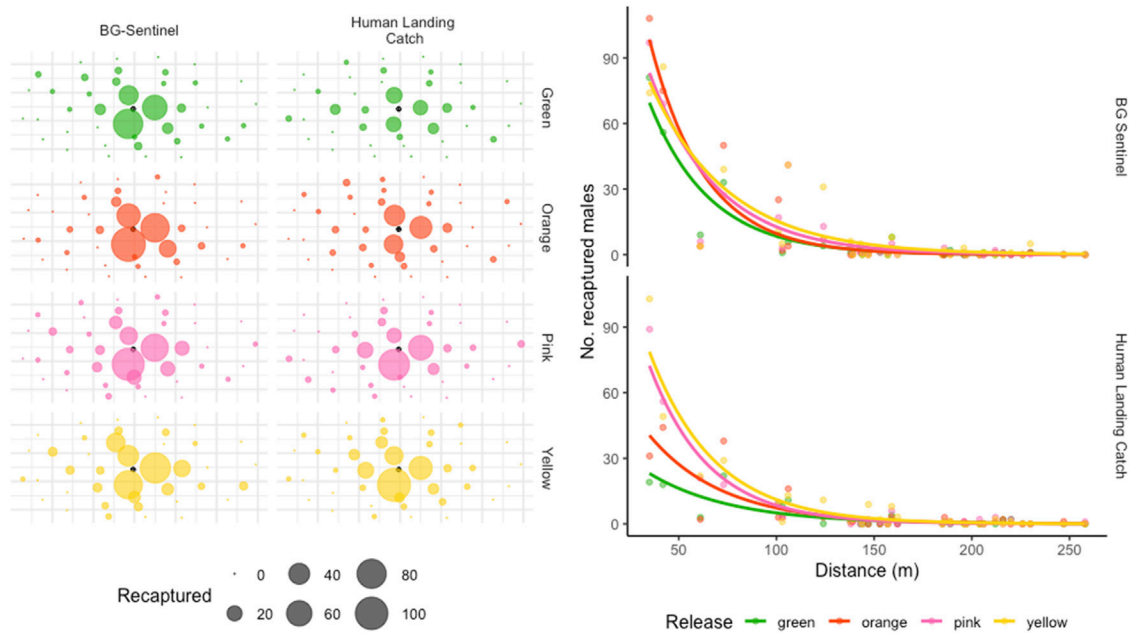

FIGURE 3 | Distribution of accumulated recaptured sterile males in different collection stations (**Figure 3A**) and dispersal pattern as a function of distance from the release point (**Figure 3B**). The black dot in **Figure 4A** represents the release point, while the size of the dots corresponds to the number of marked mosquitoes caught at each site.

time following the releases. Overall, significant variation was observed in mosquito recaptures over days of collection and the distances from the release point. The number of recaptured mosquitoes declined over time. The majority (76.47%, n = 1,443) of the total recaptured (n = 1887) mosquitoes was collected shortly after their release (the first two days post-release), 93.64% (n = 1767) within 5 days post-release and no mosquito was recaptured beyond 15 days post-release (**Figure 2**).

As sex separation before release is not always 100% efficient, there was a chance that up to 1% of females could have been released with males. In this study, 11 irradiated females that were released with males were recaptured (three with HLC and eight with BGS). Among them, one female was recaptured 247 m from the release site 1 day after release and one recaptured at 212 m 7 days after release.

Dispersal and Survival

Figure 3 shows the recapture of sterile males as a function of distance from the release point. The dispersion pattern around the released point of the recaptured population remained similar between colors and trapping methods (**Figure 3A**). The number of recaptured marked males declined with distance from the release point (**Figure 3B**). About 55.93% of sterile males were caught within a 50 m radius, 73.24% within 100 m, and 90% within 125 m from the release site (**Figure 1**). Only 1.75% was caught between 200 and 250 m radius (there were no traps beyond the 258 m). One sterile male was captured (HLC) at 258 m from the single release point 6 days after release, and one sterile male (HLC) was recaptured the day of its release at 247 m from the release point.

The dispersal pattern, estimated by the MDT, MAX, and FR within which 50% or 90% of mosquitoes are expected to disperse (FR_{50} and FR_{90}, respectively), is presented in **Table 2**. Overall, the released males were estimated to travel a mean ± SD of 93.85 ± 42.58 m (the estimated MDT over the entire period was 104.15 ± 41.95 m, 87.6 ± 32.6 m, 95.55 ± 55.5 m and 89.1 ± 40.4 (mean ± SD) m for green, orange, pink and yellow color marked males respectively). However, based on collections, the MDT was 91.2 ± 40.25 m and 96.8 ± 48.8 m for BGS and HLC, respectively. The maximum flight distance reached by 50% and 90% of the individuals was 33.01 m and 141.95 m, respectively.

The results of daily survival probability (PDS) and average life expectancy (ALE) of color-marked sterile males are presented in **Table 3**. Regardless of the color of the trapping method used, the ALE in the field varied from 3.03 to 5.76 days with average of 4.17 and 4.35 days for BGS and HLC, respectively. No statistical significance difference was found between BGS and HLC (GLM, df = 1, F = 0.213, p = 0.676). The PDS were also similar between BGS and HLC, varying from 0.72 to 0.84 with a mean of 0.79 for both methods and no statistical significance (GLM, df = 1, F = 0.02, p = 0.984). The four colors utilized showed no statistical difference both in PDS (GLM, df = 3, F = 4.437, p = 0.126) and ALE (GLM, df = 3, F = 4.215, p = 0.134).

Ratio of Sterile to Wild Males

The estimation of daily sterile to wild male ratios during the MRR is shown in **Figure 4**. The ratio varied with distance from the release point (GLM, df = 1, F = 32.3, $p < 0.05$), but no difference was observed between trapping methods (BGS and HLC) (GLM, df = 1, F = 0.0, p = 0.989). The sterile-to-wild ratio varied over

TABLE 2 | Mean distance traveled (MDT), the maximum distance traveled (MAX), and flight range of 90% (FR_{90}) and 50% (FR_{50}) of the radio-sterilized *Aedes albopictus* males in the field.

Marked sterile males	Trapping method	MDT (m)	MAX (m)	FR_{50} (m)	FR_{90} (m)
Green	BGS	99.3 (48.8)	230	27.2	131.1
	HLC	109 (35.1)	220	47.7	152.2
Orange	BGS	76.7 (23.4)	230	25.7	125.3
	HLC	98.5 (41.8)	247	40.0	141.4
Pink	BGS	85.1 (41.4)	216	28.1	138.9
	HLC	104 (69.6)	258	28.8	163.3
Yellow	BGS	98.3 (41)	230	35.6	148.2
	HLC	79.9 (39.5)	220	31.0	135.2

BGS, BG-Sentinel trap; HLC, Human Landing Catch. Values in parentheses represent the standard deviation.

TABLE 3 | Daily survival probability and the average life expectancy of the radio-sterilized *Aedes albopictus* males in the field.

Marked sterile males	Trapping method	Probability of daily survival	Average life expectancy (d)
Green	BGS	0.78	4.05
	HLC	0.80	4.55
Orange	BGS	0.76	3.64
	HLC	0.72	3.03
Pink	BGS	0.81	4.69
	HLC	0.84	5.76
Yellow	BGS	0.79	4.31
	HLC	0.78	4.06

BGS, BG-Sentinel trap; HLC, Human Landing Catch.

time (GLM, df = 1, F = 12.78, $p < 0.05$), while the trapping method had no impact (GLM, df = 1, F = 1.921, $p = 0.173$). The variation in both distance and time showed a similar trend for both BGS and HLC collection methods. The maximum sterile-to-wild male ratio obtained in the release area over time collection was 4.97 sterile for one wild male. The overall mean ratio for the entire study period was 0.45 sterile for one wild male. There was a rapid decrease in the sterile to wild male ratio (within the first week) after release and within 100 m from the release point (**Figure 4**).

Wild Male Population Size Estimation

The wild male population size in the area was estimated for the whole collection period using the Lincoln index modified version calculation that corrects for small samples and compensates for daily survival, and the results are presented in **Supplementary Table S2**. Considering only recapture data from BGS, the Lincoln index estimated the mean population size to be 72,181 males in the overall estimated area of 20 hectares equivalent to 3,609 males/ha. Meanwhile, data from HLC estimated the mean number of males to be 118,691 males (5,934 males/ha).

Field Competitiveness

In the three control sites, the natural egg fertility from June to November 2017 ranged from 90% to 100%, and no statistical difference was found among sites ($p > 0.5$). The overall natural fertility calculated in the untreated areas during the MRR study period was 98.24% ± 01.86% (mean ± SD), while in the release site the observed fertility was 74.10 ± 30.85% (mean ± SD). The Fried index estimated from 1,000 bootstraps is presented in **Figure 5**. The overall Fried Index was evaluated at 0.28% and the 95% confidence interval was [0.19–0.42].

DISCUSSION

MRR studies are commonly carried out in ecological research in a diverse range of species (McKenzie, 1974; Smith et al., 1999) to assess population size, seasonal dynamics, and dispersal (Harrington et al., 2008; Marini et al., 2010). In our study, MRR allowed us to quantify the dispersal, survival, and competitiveness of irradiated male *Ae. albopictus* in preparation for a field trial in Albania, as recommended for any method based on male release (Bouyer et al., 2020b). Two (series) sessions of MRR were performed with similar recapture rates for both BGS and HLC methods (3.93%) over 2 weeks in which the majority of males were caught within 5 days post-release. This result is consistent with the literature (Bellini et al., 2010) or showed higher recapture rates than in the study by Iyaloo et al. (2020) who found recapture rates in *Ae. albopictus* ranging from 0.8% to 1.3% over 6 days post release and the study by Caputo et al. (2021) that found a recapture rate of 1.8% in the first 6 days. Winskill et al. (2015) found a recapture rate of 0.36% for another genetic control method based on the release of insects carrying a dominant lethal gene (RIDL). Extreme meteorological conditions (temperature, relative humidity, and wind speed) can

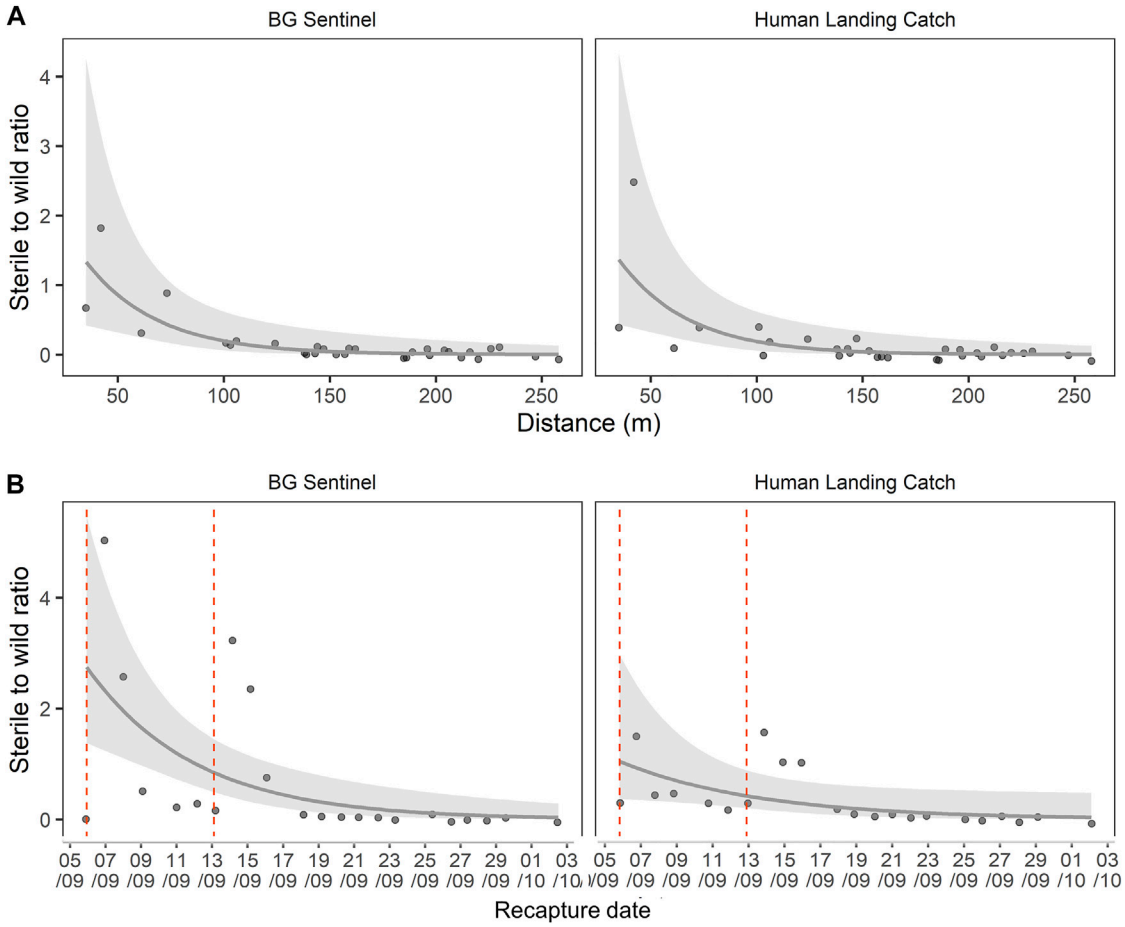

FIGURE 4 | Dynamic of the sterile-to-wild male ratio over the distance from the release point **(A)** and time elapsed since release **(B)**. Vertical dotted lines represent the release dates.

influence mosquito abundance, survival, and dispersal (Marcantonio et al., 2019). Although environmental conditions were globally suitable during this experiment, rains occurring during the second release might have caused the observed variations between the two series.

Understanding the ability of released sterile male mosquitoes to disperse in an area being targeted by a suppression strategy is crucial for predicting the required release pattern (Winskill et al., 2015). Dispersal defines the capacity of individuals to spread from a fixed or constant source (Gavriel et al., 2012). Generally, male mosquitoes may disperse to find sugar and swarms for mating. There are very few studies that examine the dispersal capacity of male mosquitoes while considering all aspects of the SIT process from production to release. Our study showed an acceptable dispersal capacity when compared to those studies (Lacroix et al., 2009; Le-Goff et al., 2019). Most of the released males were recaptured within 100 m from the release point, but the MDT was 70.78 ± 7.050 m, which is higher than the 52.8 m of RIDL male *Ae. aegypti* (Winskill et al., 2015) and close to the 83 m of irradiated male *Ae. aegypti* mosquitoes released from the ground (Bouyer et al., 2020a), both in Brazil. *Aedes* are typically short-dispersing species (Christophers, 1960), and *Ae. albopictus* males and females' active dispersal is limited to a few hundred meters (Vavassori et al., 2019). We observed a rapid decrease in the sterile to wild male ratio within the first week after release and within 100 m from the release point, suggesting the need for releasing sterile males more than once a week at a maximum distance of 100 m between release points.

Some of the released males were able to survive up to 15 days after release with a good mean survival (PDS of 0.79 and an ALE of 4.26 days), higher than the results found by Neira et al. (2014) in Panama with non-irradiated marked male *Ae. aegypti* (0.65 and 2.3 days, respectively). Trewin et al. (2021) also found a lower survival for wild male *Ae. aegypti* marked with Rhodamine B (0.55 and 1.69 days, respectively). Their MDT was, however, 295.2 m, much higher than in our study. In Brazil, irradiated male *Ae. aegypti* mosquitoes released from the ground had PDS of 0.20–0.63 (Bouyer et al., 2020a), again lower than in our study.

It is important to note that the sterile males used in our trial were imported via long-distance transportation, following long-distance transportations attempts in Europe (e.g., from CAA

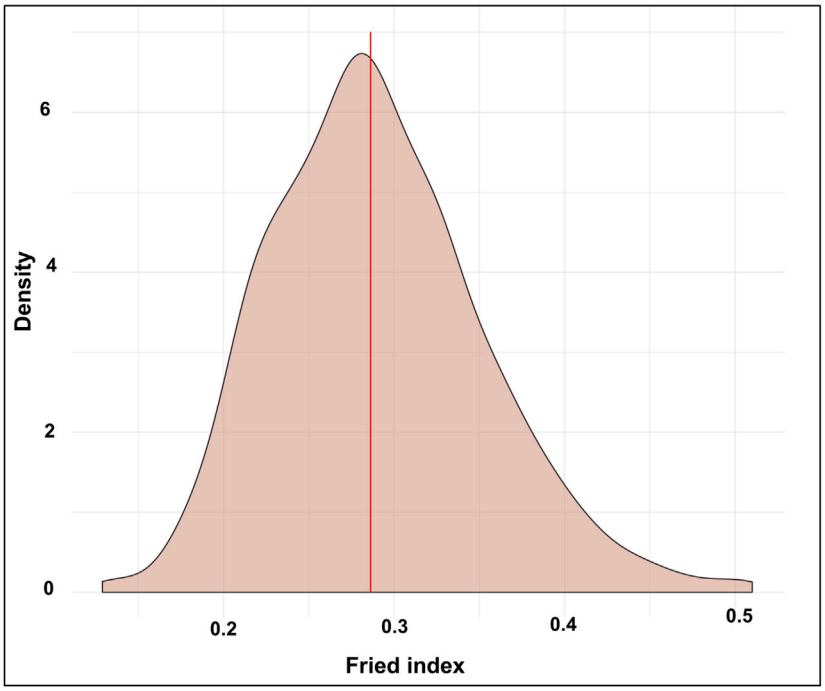

FIGURE 5 | Estimation of the Fried index from 1,000 bootstraps in the distributions of sterile to wild male ratios in traps. The density corresponds to the percentage of the simulations for a given value.

laboratory, Bologna, Italy to Germany, Montenegro, and Greece) (Balatsos et al., 2021). Despite 20% of the mortality observed during transport, the released males showed an overall good field quality. Our results show the feasibility of regional production facilities for sterile *Aedes* invasive species in support of newly invaded areas, thus reducing initial risks and costs related to the establishment of mass-rearing production units.

Knowledge of the size of the wild male population of the target area is crucial in determining the dose of sterile males required to achieve the appropriate sterile-to-wild male ratio for controlling the population effectively (Proverbs et al., 1982; Rendón et al., 2004). Here, the male population was as high as 4,700 per hectare, supporting the need of preliminary reduction of the densities with complementary methods such as breeding source reduction, door-to-door (sanitary education), and insecticide-based treatments before starting the SIT component.

We found competitiveness of 0.28 in this study, which is higher than 0.13, the observed average of several pilot trials conducted in Italian urban areas (Bellini et al., 2021). In another MRR study carried out in Brazil, the competitiveness of sterile males released using a release system mounted on an unmanned aerial vehicle (drone) was very close to our results, that is, 0.26 (95% confidence interval, 0.05–0.72) (Bouyer et al., 2020a). However, a higher competitiveness index of 0.5–0.7 with irradiated triple Wolbachia-infected male *Ae. albopictus* was observed in China (Zheng et al., 2019). Our results suggest that the quality of the released mosquitoes was still enough to run a pilot SIT trial in Albania since a competitiveness index value of 0.2 is considered the minimum acceptable threshold (Bouyer et al., 2020a).

Irradiated males are generally considered less competitive than their wild counterparts (Rendón et al., 2004; Bakri et al., 2005; Bellini et al., 2021). An overflooding ratio of 10 is an empirical target in SIT programs (Oliva et al., 2021) but may vary between species: 7:1 for some tsetse fly species (Vreysen, 2005); 60:1 for pink bollworm, *Pectinophora gossypiella* (Walters et al., 1998); 40:1 for codling moth, *Cydia pomonella* (Proverbs et al., 1982); and 100:1 for painted apple moth, *Orgyia anartoides* (Wee et al., 2005; Suckling et al., 2007). Here, we observed an average ratio of 0.45 sterile for one wild male, dropping quickly after the release day. Considering our results, this would require to approximately release 700,000 sterile males weekly in our target area of 20-ha, probably in two releases of half this number and in several release points, to cause a 74% induced sterility. This underlines again the need for a preliminary reduction of the target population using conventional methods such as breeding source reduction or to start the releases at a more appropriate period of the year when the density of the wild population is lower (Douchet et al., 2021).

Our results demonstrated that BGS + CO_2 and HLC allowed similar estimations of several parameters measured. They also showed comparable efficiency in terms of the number of mosquitoes captured (though during different collection times). To our experience, the variability of catches might greatly increase if trap stations are not chosen by experienced persons. One of the drawbacks of BGS is its price which makes it expensive to purchase and deploy since it is set for 24 h periods covering a full daily mosquito activity period. They also require power, which implies the use of a household electrical outlets or batteries. Their main advantage is to provide a more standardized

estimation of mosquito densities. Conversely, HLC is low-cost, performed only within 15 min and if possible, at the high activity periods of *Aedes*, to reduce the noise for statistical analyses. However, HLC data may suffer from variability due to differences in personal attractiveness to mosquitoes as well as individual abilities to catch mosquitoes, making this method more difficult to standardize.

This MRR study was conducted in September only (the end of mosquito season), and the total trapped area was only 250 m of ray which constitutes the main limitations of the study. However, based on the cost-effectiveness of the SIT program and the available data on such MRR studies (Caputo et al., 2021; Lacroix et al., 2009; Balestrino et al., 2022), we believe that the design limit of 250 m seems reasonable to measure the main parameters needed for testing the SIT. Although it provides important insights on the overall performance of the released sterile males, their dispersal and survival in the field as well as the size of the wild population can vary over time during the mosquito season due to variation in environmental conditions. Hence, the release strategies should take into account both the quality and the size of the wild population at different time points of the mosquito season. For reliable estimation of the wild population size over time, an MRR study should be carried out at different time points of the mosquito season (e.g., the beginning, the peak, and/or end of the mosquito season). Furthermore, another limitation of such an MRR study is the lack of non-marked controls in the experimental design. Although of importance, it was, however, not feasible in the present mark-release-recapture study since only the local strain can be released in the study area and there is no biological marker to differentiate non-marked released mosquitoes from the wild ones in order to compare their dispersal and longevity. A recent study conducted in northern Italy (Balestrino et al., 2022) on *Ae. albopictus* under the same rearing and climate conditions but released in the summer (July–August) without long-distance transportation has shown a lower recapture rate (0.27%–1.7%) and PDS 65.4% ± 11.5% to 80.0% ± 6.4%). However, they found much better flight range (FR_{50} = 76.0 ± 35.7 m; FR_{90} = 227.0 ± 74.8 m; MAX = 249.8 ± 45.2 m) and mean distance travelled (126.4 ± 44.6 to 172.9 ± 45.8 m) in comparison to our study. This difference might be due to the transportation and also to the seasonal environmental conditions, highlighting the need to carry out MRR studies at different time points. Nonetheless, a baseline data collection required before every SIT pilot trial following the phased conditional approach, coupled with this MRR study will allow a better estimation of the population abundance and size over time in Albania."

CONCLUSIONS

Overall, the results of this MRR study allowed us to estimate the minimum distance required between sterile male release points (100 m), as well as the necessary frequency (3–4 days) of releases to be used in future SIT trials in Albania. The release time in the day did not appear as a constraint. Irradiated males dispersed, survived long enough and competed well enough with their wild male counterparts to warrant the feasibility of the next phase, that is, a suppression trial. The main limitations of this study are that it was conducted only in September and should be repeated at the beginning of the mosquito season (April–June) and that the total trapped area was only 250 m of ray. However, our data still provide critical baseline information for better decision-making, designing and elaborating of appropriate planning of SIT pilot suppression studies in Albania and other countries in Europe with similar environmental conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Albania is not exposed to *Aedes* borne endemic transmission, and the operators gave their informed consent to carry out HLC after being informed of the potential risks of bites. The study was authorized by the Ministry of Health and Social Protection in Albania.

AUTHOR CONTRIBUTIONS

EV, FB, SB, LT, PK, RB, FS, DA, DP, AP, AM, JB, and WM designed the study and wrote the methodology. AP performed laboratory work for sterile male production. GS, ER, AN, TP, TG, MK, DP, NI-A, OM, RM, IP, EF, AC, and MMA performed laboratory and field work and generated data in the web platform. EV, FB, DOC, AD, PK, AB, MA, JB, and WM performed data curation and formal analysis. EV, FB, AD, DC, JB, and WM analyzed the data. EV, LT, and JB provided funding and supervised experiments. WM wrote the first draft, and all authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.833698/full#supplementary-material

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A species-specific lncRNA modulates the reproductive ability of the asian tiger mosquito

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Long non-coding RNA (lncRNA) research has emerged as an independent scientific field in recent years. Despite their association with critical cellular and metabolic processes in plenty of organisms, lncRNAs are still a largely unexplored area in mosquito research. We propose that they could serve as exceptional tools for pest management due to unique features they possess. These include low inter-species sequence conservation and high tissue specificity. In the present study, we investigated the role of ovary-specific lncRNAs in the reproductive ability of the Asian tiger mosquito, *Aedes albopictus*. Through the analysis of transcriptomic data, we identified several lncRNAs that were differentially expressed upon blood feeding; we called these genes Norma (NOn-coding RNA in Mosquito ovAries). We observed that silencing some of these Normas resulted in significant impact on mosquito fecundity and fertility. We further focused on Norma3 whose silencing resulted in 43% oviposition reduction, in smaller ovaries and 53% hatching reduction of the laid eggs, compared to anti-GFP controls. Moreover, a significant downregulation of 2 mucins withing a neighboring (~100 Kb) mucin cluster was observed in smaller anti-Norma3 ovaries, indicating a potential mechanism of in-*cis* regulation between Norma3 and the mucins. Our work constitutes the first experimental proof-of-evidence connecting lncRNAs with mosquito reproduction and opens a novel path for pest management.

KEYWORDS

Aedes albopictus, tiger mosquito, RNAi pest control, lncRNAs (long non-coding RNAs), species-specific control

1 Introduction

The remarkable progress of next-generation sequencing and genomics technologies that took place during the past 20 years revealed an unexpected world of transcribed, non-coding (nc) genomic elements that by far exceed in numbers the protein-coding transcripts (Claverie, 2005). Long non-coding RNAs (lncRNAs) represent one class of functional ncRNA transcripts, characterized by species specificity and tissue-specific

expression patterns. LncRNA transcripts are longer than 200 nucleotides, they are mainly transcribed by unique genes and most of them are subject to post-transcriptional modifications (splicing, poly-A tail, and C-cap), although they have limited or no protein-coding potential.

Various research studies in eukaryotic organisms have highlighted the role of lncRNAs in essential biological processes and different modes have been proposed regarding their action. These modes include, but are not limited to, 1) guiding or decoying transcription factors, 2) acting as scaffolds for chromatin modifying complexes, 3) functioning as sponges for miRNAs, 4) regulating post-transcriptional mRNA modifications [reviewed in (Marchese et al., 2017)].

Due to the absence of coding capacity, lncRNAs demonstrate a notable lack of nucleotide sequence conservation even among closely related species, which results in a high number of unique, species or genus specific lncRNAs across eukaryotic organisms (Pang et al., 2006; Bhartiya and Scaria, 2016). This low sequence conservation along with the aforementioned multifunctionality provide extraordinary difficulties in the development of computational tools that would predict lncRNA targets or potential modes of action. Nonetheless, despite this sequence variation, lncRNAs from different organisms may exhibit structural or functional conservation, highlighting their conserved role in essential biological pathways (Ponjavic et al., 2007; Diederichs, 2014; Tavares et al., 2019). Indeed, unlike other non-coding RNAs (e.g., miRNAs) that hybridize to their targets through sequence complementarity patterns, the functionality of lncRNAs mainly results from their secondary structure motifs (Kino et al., 2010; Ding et al., 2014; Chillón and Pyle, 2016; Smola et al., 2016).

The high sequence divergence of lncRNAs renders them as ideal candidates for the development of species-specific population control approaches against organisms of interest. Achieving species-level specificity is a point of major importance for the development of novel pest management approaches especially against insect pests, such as mosquitoes. Current insect control approaches are mainly based on chemical insecticides that pose serious threats to public health and biodiversity due to their neurotoxic action against other species, either mammals (including human) (Costa et al., 2008) or off-target insects (Desneux et al., 2007). Beneficial insects, contributing vitally to the stability of ecosystems and to agriculture, are severely harmed by the main classes of pesticides, even by those that are considered safe for humans. This issue arises due to the lack of species-specific mechanisms underlying insecticide action. Both organophosphates and carbamates target acetylcholinesterase (AChE) which is conserved among insects (Kwong, 2002), while pyrethroids target conserved voltage-sensitive sodium channels (Soderlund, 2010). Moreover, neonicotinoid pesticides which are considered non-toxic for vertebrates, act as agonists of nicotinic acetylcholine receptors (nAChRs). However, all insects are vulnerable to these pesticides due to the conserved nAChRs sequences and their critical role in signal transduction (Simon-Delso et al., 2015). In addition to the lack of species-specificity in the currently used chemical insecticides, there is also the huge and long-standing problem of insecticide resistance. The way out of this unfavorable situation is the development of novel pesticides that target alternative gene classes that could lead to more effective pest management (Sparks et al., 2021).

The Asian tiger mosquito, *Aedes albopictus*, is also the target of various control approaches, as it is a cosmopolitan vector of several lethal arboviruses, including dengue, Zika and chikungunya (Martinet et al., 2019). *Ae. albopictus* emerged from the tropical and sub-tropical regions of south-east Asia and rapidly expanded throughout the world due to its exceptional ability to adapt in different environmental conditions (Benedict et al., 2007). Its vectorial status arose especially after its connection with the major outbreaks of Chikungunya virus in 2005-07 in La Reunion (Pialoux et al., 2007) and in 2007 in Italy (Rezza et al., 2007; Angelini et al., 2008), while it was also associated with the first autochthonous cases of dengue fever in France in 2010, 2013, and 2015 (La Ruche et al., 2010; Marchand et al., 2013; Succo et al., 2016). It is certain that in the coming years its expansion will continue, and estimates indicate that by 2050 half of the world population will be exposed to disease-spreading mosquitoes, such as *Ae. albopictus*, due to climate change and global warming (Kraemer et al., 2019).

In the present study we explore the potential of targeting lncRNAs to control insect populations. LncRNAs could be used as species-specific molecular targets for the development of next-generation pesticides (e.g., RNAi pesticides (Fletcher et al., 2020)) or be part of the rapidly growing synthetic biology systems, such as SIT and gene drives (Caragata et al., 2020). The present study focuses on the investigation of lncRNAs that are related to *Ae. albopictus* reproduction, due to the significance of reproduction in population suppression approaches. The reproductive process in females is triggered by the consumption of a blood meal (BM) which activates a cascade of metabolic signaling pathways that lead to the development and production of eggs. We sought to identify lncRNAs that influence the reproductive process, aiming at the disruption of oogenesis and the reduction of mosquito fecundity and fertility.

2 Materials and methods

2.1 Mosquito rearing

An *Aedes albopictus* laboratory line was established from wild mosquitoes which were collected from the region of Thessaly, as described elsewhere (Ioannou et al., 2021), and were reared in the insectary facility of the Department of Biochemistry &

Biotechnology at the University of Thessaly. Adult mosquitoes were reared at 26 ± 1°C with a relative humidity of 60–70%, under a 14 h:10 h light/dark photoperiod. They were fed with 10% sucrose solution, while female mosquitoes were blood-fed (BF) from a human arm to initiate their gonotrophic cycles.

2.2 RNA extraction, reverse transcription and real-time PCR

Ovaries and other tissues were dissected under the microscope and their total RNA was extracted using Extrazol (BLIRT S.A., Gdańsk, PL), according to the manufacturer's instructions. The integrity of the RNA was assessed by agarose gel electrophoresis. Total RNA was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, United States) and 1 μg of RNA was reverse transcribed to cDNA by using oligo-dT primers and MMLV-RT (Invitrogen, Waltham, MA, United States), according to the manufacturer's instructions. Each biological replicate corresponds to tissues collected from individual mosquitoes. We preferred to study tissues from individual mosquitoes, rather than pooling them together, in order to be able to assess within population variability. All qPCR assays were performed in CFX96 Real-Time Thermal Cycler (Bio-Rad, Hercules, CA, United States). All amplifications were performed with two technical replicates and the relative gene expression was analyzed by using the $2^{-\Delta\Delta Ct}$ method (Livak) through the CFX Manager™ software. Specific primers to amplify the genes identified by the transcriptomic analysis were designed using PrimerQuest™Tool. Their target specificity was verified through Primer-BLAST (Ye et al., 2012) against the RefSeq mRNA database of *Aedes albopictus*. Primers that lacked any sequence homology to other transcripts were selected. Two endogenous ribosomal housekeeping genes (RpL32, RpS17) were used for the normalization of the results (Dzaki and Azzam, 2018). The average expression stability value (M-value) of the reference genes for each biological sample was determined and samples that exhibited M-value <0.5 and Coefficient of Variance <0.25 were accepted for further analysis. Primers for qPCR are shown in Supplementary Table S2.

2.3 *In Vitro* double-stranded (ds) RNA synthesis-dsRNA treatment

Target templates for *in vitro* transcription were generated using gene specific primers with the respective recognition site for T7 RNA polymerase designed by the eRNAi web platform (Horn and Boutros, 2010). Thermodynamic parameters of the primers were tested through the web platform OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). We selected primer sets that exhibited a minimum ΔG value of −9 kcal/mol for homodimer and heterodimer formation, while their hairpin structures did not exceed the primer Tm-10°C. The target specificity of the primers was assessed by Primer-BLAST (Ye et al., 2012) against the RefSeq mRNA database of *Aedes albopictus*. The off-target effect of dsRNAs (T7 amplicons) that were introduced for silencing was evaluated by examining for potential homologies of their siRNAs with other transcripts of *Aedes albopictus*. We obtained all possible 21-mers (i.e., sliding window with width = 21 and step = 1) and subjected them to remote blastn analysis (argument -task "blastn-short" for short input queries) against RefSeq mRNA database of *Aedes albopictus*. We proceeded with dsRNAs/siRNAs that did not contain 21-mers matching any other transcript of *Aedes albopictus,* other than their lncRNA target. dsRNA was synthesized by using the MEGAscript RNAi kit (Ambion, Austin, TX) from a dsDNA template by incubating at 37°C for 16 h. dsDNA was produced by PCR with cDNA template and gene-specific primers with the T7 RNA polymerase promoter (TAATACGACTCACTATAGGG) attached to their 5' end. dsRNA was purified with standard phenol/chloroform protocol, its integrity was measured by agarose gel electrophoresis and its concentration was quantified by the Q3000 Spectrophotometer (Quawell, San Jose, CA). Green fluorescent protein (GFP) dsRNA was used as a control. For silencing with each one of the Norma genes, inseminated *Ae. albopictus* adult females were used. The mosquitoes were collected 5 days after eclosion and were anesthetized on CO_2. Then, 64.4 nl of dsRNA solution (5,000 ng/μl) was injected into their thorax using the Nanojet II microinjector (Drummond, Birmingham, AL), under a Leica™ stereoscope. At least 25 individual females (i.e., biological replicates) were used for silencing with each Norma gene. Two controls were used for comparison: one was non-injected females (untreated) and the other was GFP-dsRNA injected females (anti-GFP). Both control populations were reared under the same conditions with the Norma-dsRNA injected ones. Primers for dsRNA production are shown in Supplementary Table S2.

2.4 Phenotypic assays

Five-day-old mosquitoes injected with either GFP-dsRNA or Norma3-dsRNA were transferred to Bugdorm™ 17.5 × 17.5 × 17.5 cm cages (MegaView Science Co., Talchung, Taiwan) immediately after injection, where they were fed with 10% sucrose solution for 24 h to recover. An additional sample of same age, non-injected mosquitoes (untreated) was also maintained. The mosquitoes were left to starve for 12 h and subsequently they were blood-fed (36 h post-injection). All blood meals took place in the same timeframe, between morning and noon, to avoid the influence of circadian variability among the replicates. Fully engorged, blood-fed mosquitoes were reared in the presence of 10% sucrose.

2.4.1 Ovarian measurement

Ovaries were dissected 60 h post-blood meal by detaching the soft cuticle between the fifth and sixth abdominal segment and pulling the terminal segments with fine forceps onto a drop of phosphate buffered saline (PBS). Pictures of the ovaries were captured with a Leica™ stereoscope (Leica Microsystems, Wetzlar, Germany) and the length of the long axis of the ovarian follicle was measured by FIJI software (Schindelin et al., 2012).

2.4.2 Oviposition

Four days after blood feeding individual mosquitoes were placed in polystyrene fly vials that contained a filter paper attached to a wet cotton ball and were left into the vial for 2 days in the insectary to lay their eggs. Additional moisture was added regularly to the vials to keep the filter paper wet. Two days later, mosquitoes were removed from the vials and the total number of eggs deposited by each individual mosquito was counted under a stereoscope. Mosquitoes that deceased during the process were excluded from the analysis.

2.4.3 Hatching assay

Laid eggs were dried and then stored into sealed petri dishes that contained a wet cotton ball as a source of humidity. Each petri dish hosted the eggs laid by one individual mosquito. Three days (72 h) post egg laying, 30 ml of hatching broth, that was prepared as described elsewhere (Maïga, 2017), were added into each petri dish. Eggs that were stored inside the petri dishes were incubated with the hatching broth for 14 days. Hatching broth was freshly replaced every 5 days. The long 14-day period was preferred because shorter periods led to reduced hatching of the eggs, probably due to diapause effect. Emerged larvae from each egg batch were counted daily and were removed from petri dishes. Finally, the hatch rate was estimated as the total number of emerged larvae divided by the total number of laid eggs:

$$hatch\ rate = \frac{emerged\ larvae}{laid\ eggs} \times 100\%$$

2.4.4 Bleaching assay

The filter papers that contained the eggs that were laid by individual dsRNA-injected females were collected, placed separately into petri dishes and left under moisture for 72 h to complete their embryogenesis. Then they were dechorionated in order to visualize their internal segments, based on a clarification methodology described elsewhere (Trpiš, 1970). A fresh Trpiš solution (3gr $NaClO_2$, 2 ml glacial acetic acid, in 1 L distilled H_2O) was prepared for each experiment and was added into the petri dishes that hosted eggs that were collected from mosquitoes injected with dsRNA against Norma3 or GFP. The eggs were incubated with Trpiš solution for 40 min at room temperature and then were immediately washed with PBS and placed gently with a soft brush into a drop of PBS under a Leica™ stereoscope (Leica Microsystems, Wetzlar, Germany) for visualization.

2.5 RNA-seq data pre-processing and primary analysis

Raw FASTQ files from a detailed, publicly available, developmental transcriptomic dataset by Gamez and colleagues were retrieved from the Sequence Read Archive (SRA) study entry SRP219966 (Gamez et al., 2020), using SRA toolkit. Quality assessment, identification of adapters and overrepresented contaminants was performed by employing FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the EMBL-EBI's Minion application (http://www.dev.ebi.ac.uk/enright-dev/kraken/reaper/src/reaper-latest/doc/minion.html). Quality trimming and adapter trimming of reads was performed with cutadapt. Pre-processed FASTQ files were mapped to the GCF_001876365.2_canu_80X_arrow2.2 *Ae. albopictus* genome assembly utilizing STAR splice-aware aligner (v2.7.9a) (Dobin et al., 2013) using the ENCODE standard options provided in the STAR manual. The respective transcript annotation file, derived from the NCBI Eukaryotic Genome Annotation Pipeline (Gnomon gene predictions), was also provided (--sjdbGTFfile argument) to effectively account for known splice junctions, while the genome index was accordingly parameterized to the read length (--sjdbOverhang 49). Transcript-level expression was calculated with RSEM v1.3.1. (Li and Dewey, 2011).

2.6 LncRNA annotation

Accession numbers with the "XR_" prefix, annotated as non-coding RNA (ncRNA) were retained from the GCF_001876365.2 gene models. In order to obtain estimates of the transcripts' coding potential, CPC2 (Kang et al., 2017) and FEELnc (Wucher et al., 2017) tools were employed. Both tools were executed at default settings (in the absence of any known lncRNAs, FEELnc "--mode = shuffle" method was chosen and the "XM_" (mRNA) transcripts were also provided as input for training). Transcripts were queried locally with BLASTn v2.13.0 (Altschul et al., 1990), at default settings, against Reference RNA Sequences (RefSeq_rna) from all Hexapoda species (Taxonomy ID: 6960). Each transcript was annotated with respect to the number of species it presented hits to, besides *Ae. albopictus*. FEELnc classifier was also used to characterize all "XR_" transcripts as genic and intergenic, according to their genomic localization and strand, respective to other transcripts. Lastly, RSEM-derived counts from all analyzed datasets in Section 2.6 were TMM-normalized (Robinson and Oshlack, 2010), log_2-

transformed and utilized to obtain *tau* (τ) tissue specificity indices (Yanai et al., 2005) (https://github.com/roonysgalbi/tispec). For a transcript x with expression x_i across n tissues/contexts, *tau* is obtained using its expression normalized by its maximal expression as follows:

$$tau = \frac{\sum_1^n (1 - \hat{x}_i)}{n - 1};\ \hat{x}_i = \frac{x_i}{\max(x_i)_{1 \le i \le n}}$$

After deriving the global *tau* for a transcript, its per-context specificity fraction was calculated by multiplying *tau* with the maximal-normalized expression metric $\hat{x}_i$. "XR_" transcripts were annotated with their context (i.e., developmental stage, tissue and time-point) *tau* specificity index, as well as per-context fraction. Heatmaps were created using R package pheatmap, using Euclidean distance as a metric and complete-linkage hierarchical clustering where applicable. Annotation results are provided unfiltered in Supplementary Data S1.

2.7 Norma3 expression correlation and clustering analysis

For correlation analysis, samples where Norma3 presented non-zero expression (Transcripts-Per-Million, TPM units) were selected and transcripts presenting zero TPM across all samples were filtered out. Pearson correlation testing and False Discovery Rate adjustment of *p*-values were conducted in R. Clustering analysis was performed by providing TPM values to DPGP (McDowell et al., 2018), which applies Dirichlet Process to nonparametrically determine the optimal number of expression trajectory clusters and Gaussian Process to model the trajectories of expression through time. Following DPGP recommendations, a limited list of transcripts was subjected to clustering analysis instead of the whole transcriptome. For that purpose, read counts from post-blood meal (PBM) ovary samples (12, 24, 36, 48, 60, and 72 h) were imported in R. In the absence of replicates, 12–24 h, 36–48 h and 60–72 h time points were grouped into "early", "middle" and "late" groups respectively. EBSeq (v1.30.0) (Leng et al., 2013) was utilized to assign transcripts to patterns of differential expression, at a relaxed posterior probability ≥75%. DPGP was executed at default settings.

2.8 Statistical analysis

Data were presented as mean ± SEM of independent biological replicates, unless otherwise noted. Distribution patterns of the samples were evaluated through Shapiro-Wilk test (Shapiro and Wilk, 1965) and those populations that followed normal distribution were analyzed by two-tailed unpaired Student's t-test. Samples that did not pass normality test were analyzed by nonparametric two-tailed Mann-Whitney *U* test (Mann and Whitney, 1947). *p*-values of ≤0.05 were considered as significantly different. All analyses were performed through GraphPad Prism 8 software and all values are displayed in Supplementary Data S3.

3 Results

3.1 Annotation of predicted non-coding RNA in *Ae. albopictus*

In order to query for *Ae. albopictus* lncRNAs that are potentially implicated in mosquito reproduction, we initiated our computational analysis on the respective NCBI gene models, which contain 8571 transcripts that are predicted to belong to the ncRNA class ("gbkey = ncRNA"). Bioinformatics approaches were adopted to annotate further these RNAs regarding their coding potential, species specificity, genomic localization, as well as their context-expression specificity within *Ae. albopictus*.

Coding potential estimates of the predicted ncRNAs were calculated with CPC2 and FEELnc. CPC2 (Kang et al., 2017) is a well-known species-agnostic Support Vector Machine model that makes use of Fickett score, open reading frame (ORF) length and integrity and isoelectric point to predict coding potential. In contrast, FEELnc (Wucher et al., 2017) accepts as input known coding and non-coding transcripts from a species of interest and trains a species-aware coding potential model (Random Forests) using as predictors the ORF coverage of transcripts, k-mer composition (1-, 2-, 3-, 6-, 9-, and 12-mers) and transcript length. When known non-coding RNA sequences are not available, under shuffle mode, FEELnc shuffles the provided protein coding sequences but preserves 7-mer frequencies, to create a faux set of non-coding transcripts to use in model training-testing, a method shown to fare adequately in benchmarks against real lncRNAs (Wucher et al., 2017). The FEELnc model output is a score between 0 (i.e., no coding potential) and 1 (coding potential), while its optimal cutoff point that maximizes sensitivity and specificity is calculated by means of 10-fold cross validation. CPC2 annotated 8289 "ncRNA" transcripts as non-coding and 282 as potentially coding. FEELnc classification yielded 8096 and 475 transcripts labeled as non-coding and coding respectively, at an optimal cutoff point of 0.43 (Figure 1A). CPC2 and FEELnc predictions were juxtaposed with each other, highlighting 7,900 and 86 transcripts (92 and 1% of total) concordantly classified as non-coding and coding RNA respectively, and identifying 589 predictions (7% of total) that were discrepant between the two approaches (Figure 1B, Supplementary Data S1).

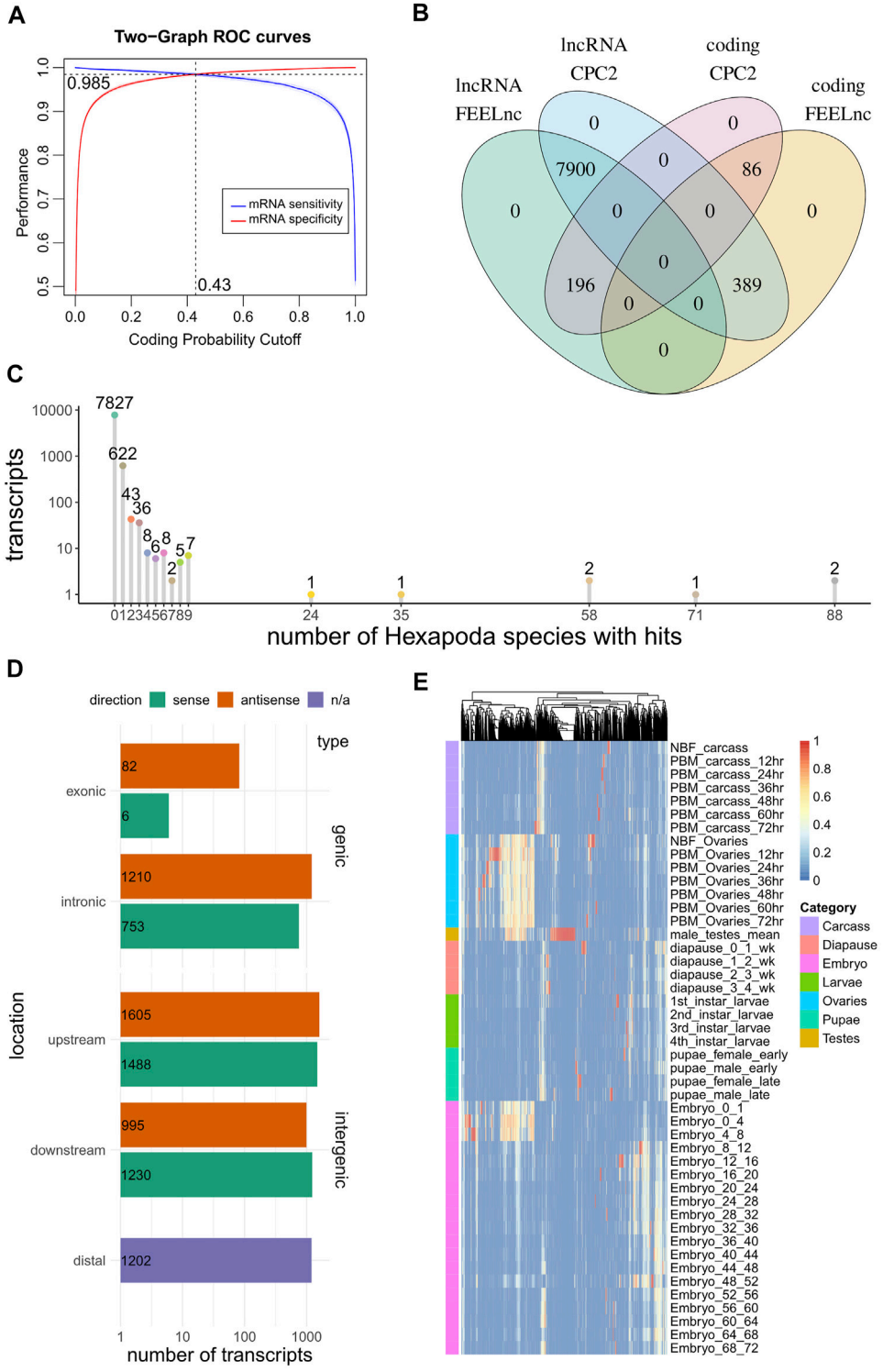

FIGURE 1
Computational analysis of RNAs predicted to be non-coding via automated NCBI analysis. **(A)** A coding potential prediction model (FEELnc Random Forests) was trained specifically on *Ae. albopictus* sequences. The optimal cutoff to discriminate coding from non-coding sequences was set at the point where sensitivity and specificity was maximized (10-fold cross validation). **(B)** Overlap of coding potential predictions between FEELnc and CPC2 models. **(C)** Depiction of number of transcripts presenting hits to other Hexapoda species. Transcripts are tallied by the number of species in which they presented BLASTn hits (*x*-axis). **(D)** Genomic localization of lncRNAs, per type (genic/intergenic), subcategory (exonic/intronic/upstream/downstream/distal (i.e., >100 kb afar from other transcripts) and strand relative to close/overlapping elements (sense/antisense). **(E)** Heatmap of per-context fractions for all lncRNAs presenting *tau* > 0.5.

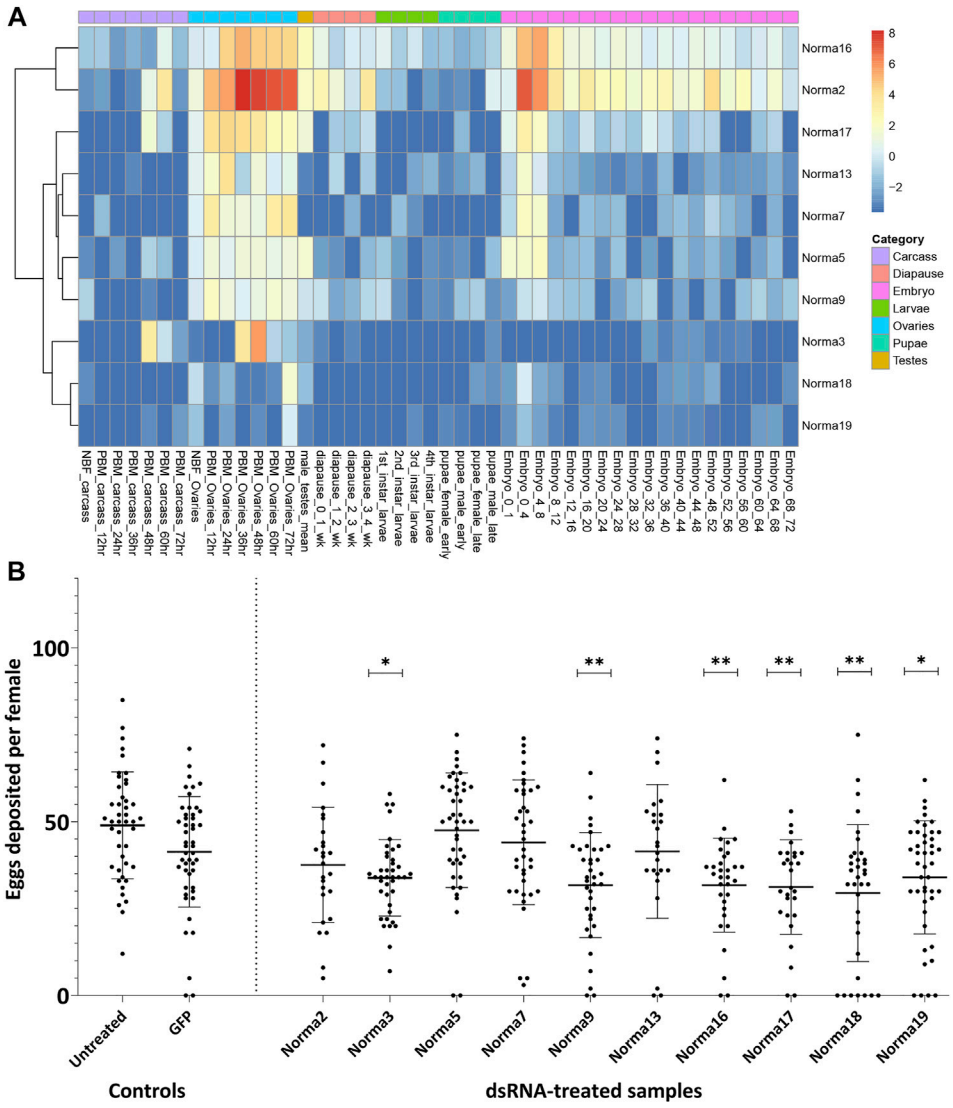

FIGURE 2
Expression of Norma transcripts across mosquito developmental contexts and oviposition rate change after dsRNA-treatment against them. **(A)** Heatmap of Norma expression levels in carcass, ovary, testis, diapause, larva, pupa and embryo samples from a publicly available transcriptomic dataset by Gamez *et al.* Expression is depicted as $\log_2$-transformed TMM-normalized counts. **(B)** Impact of dsRNA-treatment, against each Norma separately, in oviposition rate. Each dot corresponds to the number of eggs that were laid individually by each female mosquito treated with dsRNA against 10 Norma or GFP dsRNA-treated mosquitoes and untreated control. Each sample contained at least 25 mosquitoes (biological replicates) and an unpaired two-tailed student's t-test was conducted to assess the statistical significance of the results, by comparing the anti-GFP sample (control) with each anti-Norma sample. Treatment with dsRNA against Norma3, 9, 16, 17, 18, and 19 displayed statistically significant differences that may represent the influence of those genes on oviposition. Results are presented as mean ± SD. *:*p*-value≤0.05, **:*p*-value ≤0.01.

In order to identify species-specific lncRNAs, transcript homology queries were performed (local BLASTn) against all Hexapoda Reference RNA sequences. Each *Ae. albopictus* "ncRNA" transcript was annotated with respect to the number of other species it presented hits against. Out of the total transcripts, 7,827 (91%) were found to exhibit no similarity with RNAs of any other Hexapoda species (Figure 1C).

FEELnc was also utilized to annotate transcripts' localization with regard to transcripts annotated as protein coding (Figure 1D). In total, 2051 instances (24%) were found to overlap protein coding transcripts in sense (9%) or antisense (15%) orientation. The remaining 76% was divided among intergenic transcripts that presented non-overlapping neighboring genes within 100 kb of their loci (5,318 transcripts, 62%) and those that were found to exist in

genomic regions not harboring other genes (annotated as "distal" intergenic, 1,202 instances, 14%).

Finally, in order to obtain expression metrics with regard to these ncRNAs in discrete developmental stages and tissues, a publicly available developmental transcriptomic dataset of *Ae. albopictus* (Gamez et al., 2020) was analyzed from scratch (SRA accession number SRP219966). Briefly, the dataset captured expression estimates from adult ovaries of non-blood-fed (NBF, fed with a 10%-sucrose solution) and post-blood meal (PBM, at 12-24-36-48-60-72 time-points) insects, carcasses (i.e., female bodies without the ovaries, also NBF and PBM at the same time-points), adult testes, diapause, larvae, pupae and embryo samples at numerous time-points. In the absence of replicates (with the exception of testes which were in duplicate), no attempt to assess differential expression was performed. Instead, $\log_2$-transformed TMM-normalized counts were used to obtain *tau* indices (Yanai et al., 2005), which have been recently shown to perform consistently as a tissue specificity metric (Kryuchkova-Mostacci and Robinson-Rechavi, 2017). Briefly, *tau* constitutes a per-transcript tissue specificity unit ranging from 0 (no specificity) to 1 (highest specificity), while the fractions of each tissue that contributed to calculation of *tau* can also be obtained. Within the current dataset that is composed of a number of distinct tissues, developmental stages and time-points, we denoted that *tau* be regarded as a context-specificity index. The fractions of 3,119 transcripts exhibiting *tau* > 0.5 are presented as a heatmap (Figure 1E) for all available contexts, outlining the existence of numerous instances of intermediate-to-high specificity in ovary contexts (i.e., yellow-to-red transcripts within ovary samples).

We focused our attention on 984 transcripts that were 1) annotated as having no/limited coding potential by both CPC2 and FEELnc, 2) presented no sequence similarity to known transcripts of other Hexapoda and 3) exhibited *tau* > 0.5 and *tau fraction* > 0.5 in at least one PBM ovary time-point. Aiming to experimentally scrutinize a limited number of lncRNAs, out of this subset we selected 10 ovary-specific lncRNAs that were upregulated upon blood-feeding and presented limited or no expression in other developmental samples (Supplementary Table S1; Figure 2A). We termed these lncRNAs Norma (NOn-coding RNAs in Mosquito ovAries).

3.2 Phenotypic impact of norma genes silencing

In order to clarify the potential role of the 10 Norma genes, we evaluated their phenotypic impact in reproduction through RNAi silencing. Given their expression pattern, we reasoned that knocking down Norma genes would mostly impact mosquito oviposition. To assess this, we generated *in vitro* dsRNA against each of the ten Norma lncRNAs which we injected into 5-day old inseminated adult females. We then provided a blood meal to the injected mosquitoes and monitored them for oviposition. We counted the number of eggs that were laid by each individual mosquito treated with any of the ten Norma-dsRNA (anti-Norma) and compared oviposition with a GFP-dsRNA sample (anti-GFP). A non-injected, blood-fed sample (untreated) was also present in the assay to monitor the effect of the environmental conditions. We observed that females injected with six different Norma-dsRNAs laid fewer eggs compared to GFP-dsRNA injected control, indicating the potential influence of silencing the corresponding lncRNAs to reproduction (Figure 2B). Specifically, dsRNA against Norma3, Norma9, Norma16, Norma17, Norma18 & Norma19 exhibited statistically significant reduction of oviposition rates compared to the GFP control (p-value ≤0.05). We focused our downstream analyses on Norma3 because it also presented some further striking features: an ovary-specific expression profile along with a sharp upregulation pattern in the post-vitellogenic time-points. Other lncRNAs that influenced oviposition (Norma9, Norma16, Norma17, Norma18, and Norma19) will be the subject of a future investigation.

3.3 Norma3 expression analysis and RNAi-knockdown

Initially, we determined the detailed expression profile of Norma3 by examining its stage- and tissue-related patterns in samples collected from our laboratory mosquito strain. We collected ovaries at the same time-points as the ones that were described in the RNA seq dataset that we analyzed. Specifically, we collected ovaries from NBF and PBM (12, 24, 36, 48, 60, and 72 h) time-points. In addition, we dissected and stored other tissues from the same time-points (carcass, midgut, Malpighian tubes, and head). Norma3 exhibited basal expression in NBF and early PBM samples, while its expression abruptly increased >2000-fold in 60 h-PBM ovaries, compared to the expression in NBF ovaries, and then significantly dropped to 200-fold in 72 h-PBM ovaries, compared to NBF (Figure 3A). At the same time-point (60 h PBM), Norma3 displayed basal expression in all other examined tissues (carcass, midgut, Malpighian tubes, head) (Figure 3A). The expression pattern was in accordance with publicly available RNA seq data (Gamez et al., 2020). Subsequently, we estimated the efficiency of Norma3 silencing in the ovaries of female mosquitoes injected with Norma3-dsRNA (anti-Norma3), compared with mosquitoes injected with GFP-dsRNA (anti-GFP). We collected ovaries from individual mosquitoes injected with either Norma3-dsRNA or GFP-dsRNA 60 h PBM and measured the effect of RNAi on the expression levels of Norma3. Results showed an average expression drop of >50% in anti-Norma3 replicates compared to anti-GFP (Figure 3B), which presented adequate statistical

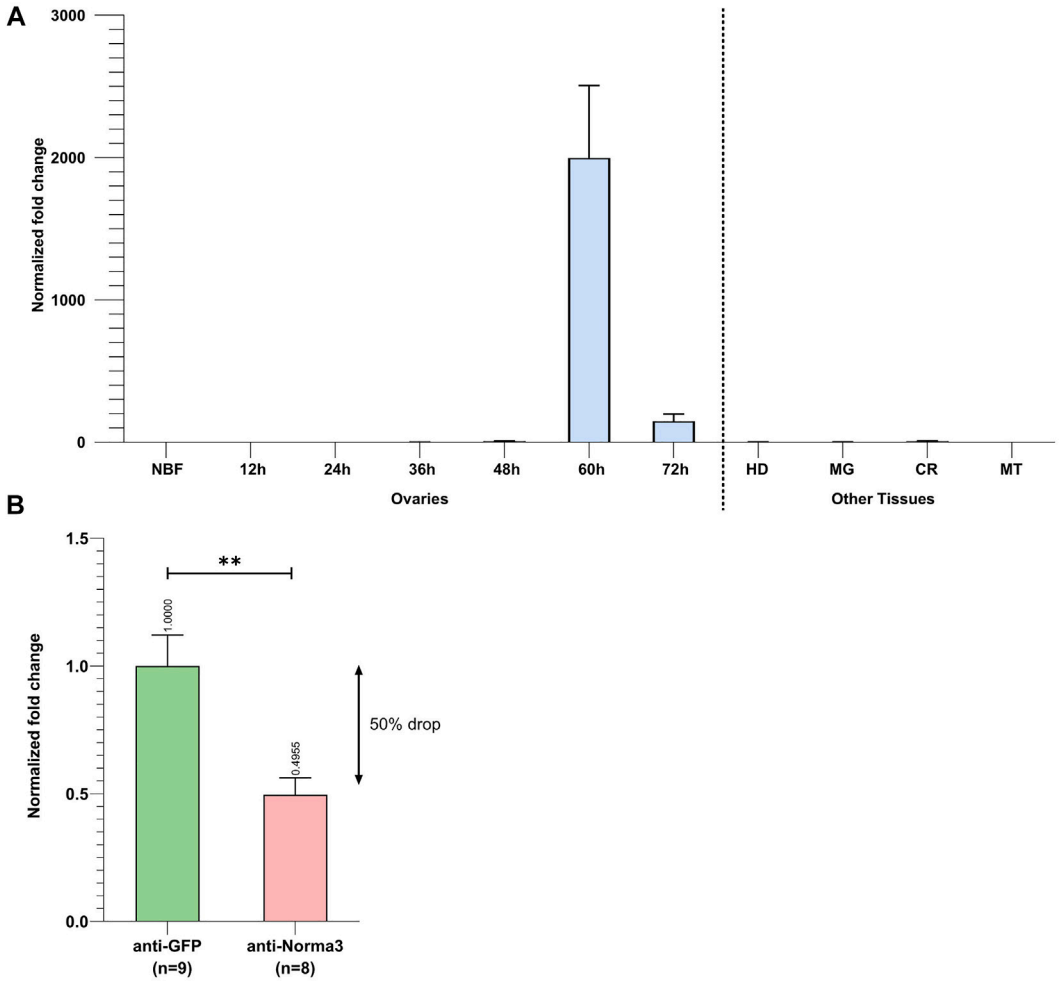

FIGURE 3
Spatiotemporal expression of Norma3 and its silencing efficacy. **(A)** Expression profile of Norma3 among non-blood fed (NBF) ovaries, post-blood meal (PBM) ovaries and other tissues collected 60 h PBM. Ovaries of blood-fed mosquitoes were collected every 12 h upon a blood meal, ranging from 12 to 72 h PBM when egg development is completed. Ovary samples (NBF & PBM) contained ovaries collected from 5-6 individual mosquitoes (biological replicates). Norma3 exhibits a basal expression in NBF, 12, 24, 36, and 48 h PBM ovaries, which abruptly increases at 60 h PBM leading to a fold change of >2,000 at 60 h PBM ($\bar{x}$ = 2,387 ± 455.8, n = 5) which drops to 200-fold in 72 h-PBM ($\bar{x}$ = 199.6 ± 46.6, n = 6), compared to NBF ovaries ($\bar{x}$ = 1 ± 0.27, n = 6). The other tissues that are presented were collected 60 h PBM. OV = Ovaries, HD = Head, MG = Midgut, CR = carcass, MT = Malpighian tubes. Each sample contained tissues collected from three individual mosquitoes (biological replicates). Fold change is presented relatively to NBF ovaries. All values were normalized with ribosomal genes RpL32 & RpS17 and are presented as mean ± SEM. **(B)** Relative quantification of Norma3 expression in replicates of the anti-Norma3 dsRNA vs. anti-GFP dsRNA samples. Ovaries were collected 60 h PBM, the time point when Norma3 peaks its expression. Each sample contains 8-9 biological replicates. Average expression of Norma3 in anti-GFP replicates was set as 1 ($\bar{x}$ = 100 ± 11.4, n = 9) and the overall expression drop of anti-Norma3 replicates, compared to anti-GFP, was measured to 50% ($\bar{x}$ = 49.5 ± 6.2, n = 8, p = 0.0031). All values were normalized with ribosomal genes RpL32 & RpS17 and are presented as mean ± SEM, **:p-value ≤0.01.

significance (Student's t-test, p = 0.0031) to support our experimental pipeline.

3.4 Impact of Norma3 silencing on reproduction

To more deeply characterize the impact of Norma3 silencing on *Ae. albopictus* reproductive ability, we examined various phenotypic traits that are connected to reproduction. First, we looked at the ovary morphology at 60 h PBM by measuring the long axis of the ovoid follicle of ovaries dissected by individuals of the anti-Norma3 dsRNA and anti-GFP dsRNA samples. We repeated the dissection process three times and detected that ovaries of anti-GFP sample presented an average length of 293.6 nm, while ovaries of anti-Norma3 presented a significantly lower length of 240 nm (Mann-Whitney test, p = 0.003) (Figure 4A). Interestingly, anti-Norma3 sample included

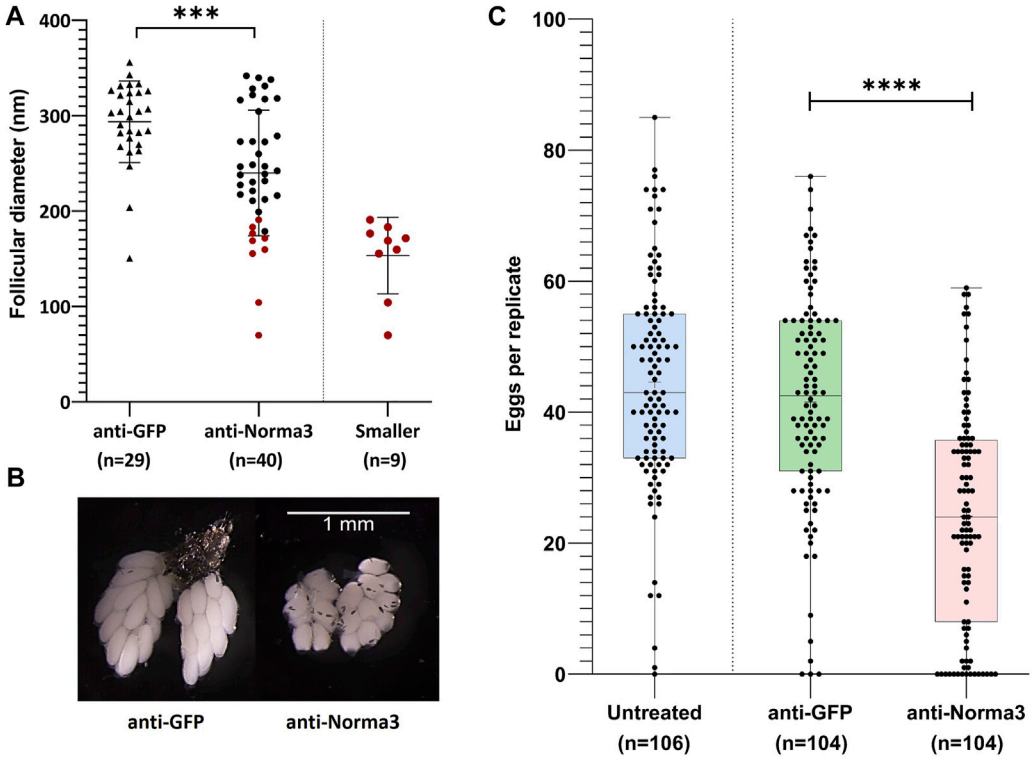

FIGURE 4
Anti-Norma3 treatment leads to abnormal maturation of ovaries and reduced oviposition. **(A)** Comparison of the length (nm) of the long axis on the ovoid follicles from ovaries obtained from mosquitoes injected with anti-GFP and anti-Norma3 dsRNA. Follicles of the anti-Norma3 sample have a smaller size ($\bar{x}$ = 240 ± 65.1, n = 40) (p = 0.0003), compared to the anti-GFP ($\bar{x}$ = 293.6 ± 42, n = 29). Smaller cohort is a subgroup of the anti-Norma3 sample that includes highlighted smaller replicates ($\bar{x}$ = 153.4 ± 37.8, n = 9). **(B)** Representative ovaries dissected 60 h PBM from the anti-GFP and the anti-Norma3 samples. Smaller follicle size and nurse cells are evident in smaller anti-Norma3 ovaries. **(C)** Number of deposited eggs per individual mosquito of untreated ($\bar{x}$ = 44.6 ± 16.3, n = 106), anti-GFP ($\bar{x}$ = 41.5 ± 16.4, n = 104) and anti-Norma3 ($\bar{x}$ = 24.1 ± 16.7, n = 104) samples. Each dot corresponds to the number of eggs that were laid individually by each female mosquito treated with dsRNA against Norma3 or GFP and untreated control. Each sample contained more than 100 mosquitoes (biological replicates) and an unpaired two-tailed student's t-test was conducted to assess the statistical significance of the results, by comparing the anti-GFP (control) with anti-Norma3 sample. Anti-Norma3 exhibits statistically significant reduced oviposition. All values are presented as mean ± SD. Error bars include values from min to max. ***: p-value ≤0.001, ****: p-value ≤0.0001.

several much smaller ovaries that had a shorter follicular diameter (Supplementary Data S3), while there was evidence of the presence of nurse cells along with their oocyte, indicating their delayed development (Figure 4A,B, Supplementary Figure S1B). Nine anti-Norma3 smaller ovaries that displayed a mean length of 153.4 nm were collected and stored for further analysis.

Afterwards, we estimated the effect of the Norma3-dsRNA on oviposition by counting the number of eggs that were laid individually by each female of anti-Norma3, anti-GFP and untreated samples. Mosquitoes of the untreated control laid an average of 44.6 eggs, while mosquitoes of the anti-GFP control laid an average of 41.6 eggs. On the other hand, mosquitoes of the anti-Norma3 treatment laid an average of 24.1 eggs. Interestingly, 14% (n = 15) of the anti-Norma3 replicates laid zero eggs, while 26% (n = 27) laid fewer than 10 eggs. In contrast, 3% (n = 3) of the anti-GFP and 1% of the untreated samples (n = 1) laid 0 eggs, while 6% (n = 6) of the anti-GFP and 3% of the untreated samples (n = 3) laid fewer than 10 eggs (Supplementary Data S3). The reduction of the average oviposition rate between anti-Norma3 and anti-GFP was 43% and exhibited high statistical significance (Student's t-test, p < 0.0001) (Figure 4C).

Subsequently, we addressed the hatch rate of untreated, anti-GFP and anti-Norma3 samples. We counted the number of larvae that emerged from the eggs that were laid by each individual mosquito of the studied samples and we divided by the total amount of eggs that were laid by each mosquito. Untreated mosquitoes displayed an average hatch rate of 79%, anti-GFP mosquitoes presented a similar hatch rate 78%, while anti-Norma3 mosquitoes had a much lower rate of

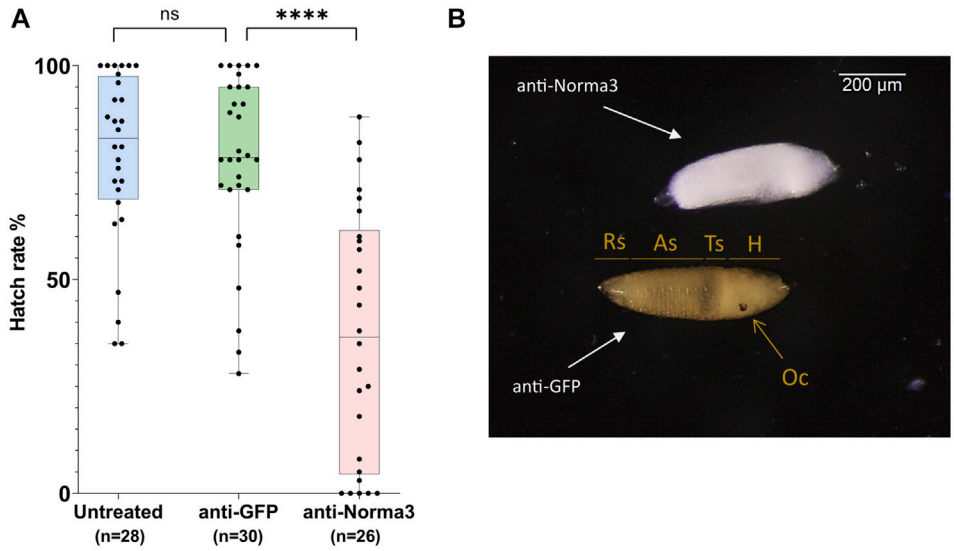

FIGURE 5
anti-Norma3 treatment reduces hatch rate and disrupts regular embryonic development. **(A)** Comparison of hatchability of eggs laid by individual females of untreated ($\bar{x}$ = 78.9 ± 19.9, n = 28), anti-GFP ($\bar{x}$ = 77.9 ± 20.2, n = 30), and anti-Norma3 ($\bar{x}$ = 36.9 ± 28.9, n = 26) samples. No statistical significance was observed between the untreated and anti-GFP samples, while high significance was detected between anti-GFP and anti-Norma3 samples ($p < 0.0001$, Mann-Whitney test). All values are presented as mean ± SD. ****: p-value ≤0.0001 **(B)** Effect of anti-Norma3 treatment in eggs that were dechorionated with Trpiš solution. Two representative embryos are displayed. Anti-GFP embryo presents regular development as based on the presence of respiratory siphon (Rs), eight abdominal segments (As), thoracic segments (Ts), head (H) and ocelli (Oc). On the contrary, anti-Norma3 does not present any of those structures.

37%. (Figure 5A). The difference of the hatch rate between anti-GFP and anti-Norma3 was about 53% and presented high statistical significance (Mann-Whitney test, $p < 0.0001$).

Finally, we attempted to visualize possible larval defects in the anti-Norma3 treated sample. For this, we rendered 72 h eggs transparent by bleaching and observed the larvae under the microscope. We investigated the entire batches of eggs/embryos laid by each mosquito treated with dsRNA either against Norma3 or GFP and detected significant changes. There was a constant percentage of 75-85% that presented the deficient phenotype in anti-Norma3 sample, while the respective percentage of anti-GFP embryos was 10-15%. We dechorionated the embryos laid by 10 anti-Norma3 and 10 anti-GFP mosquitoes to validate the result. Representative images of dechorionated embryos that were laid by different mosquitoes of each sample and displayed the characteristic phenotype are presented in Figure 5B and Supplementary Figure S2. In anti-GFP embryos the eight abdominal segments, the thoracic segments, the respiratory siphon, the head and the ocelli were clearly visible indicating regular development of the embryo. On the other hand, anti-Norma3 embryos did not present any of the anticipated normal structures; instead, they exhibited a defective appearance of an undifferentiated mass (Figure 5B, Supplementary Figure S2B).

3.5 Regulatory interplay of Norma3 with neighboring mucins

Since lncRNAs often regulate coding genes in their genomic neighborhood (Rinn and Chang, 2012; Engreitz et al., 2016; Joung et al., 2017; Khyzha et al., 2019; Xing et al., 2021), we investigated the possible association of Norma3 with protein-coding genes in its vicinity. We returned to the available transcriptomic study containing NBF and PBM ovary datasets (Gamez et al., 2020) and assessed the correlation of Norma3 expression against the rest of the transcriptome. Within the region from 100 kb upstream to 100 kb downstream of Norma3, we identified 8 annotated transcripts (i.e., 4 mucins, 1 venom protein, and 3 chymotrypsin inhibitors) which all exhibited robust positive correlation with Norma3 (Pearson correlation coefficient >0.96, maximum FDR = 2.17e-9) (Supplementary Data S2). Subsequently, we reduced the transcriptome to transcripts which were more likely to present any change in ovaries among early (i.e., 12–24 h), middle (36–48 h) and late (60–72 h) time points (posterior-probability EBSeq >75%). This transcript set (n = 10,314) was subjected to clustering analysis based on their expression over the entire time course (i.e., NBF, 12, 24, 36, 48, 60, and 72 h PBM). Norma3 was grouped together with 114 other genes (111 protein-coding and 3 long non-coding) in a cluster of genes that was not expressed in NBF and early PBM

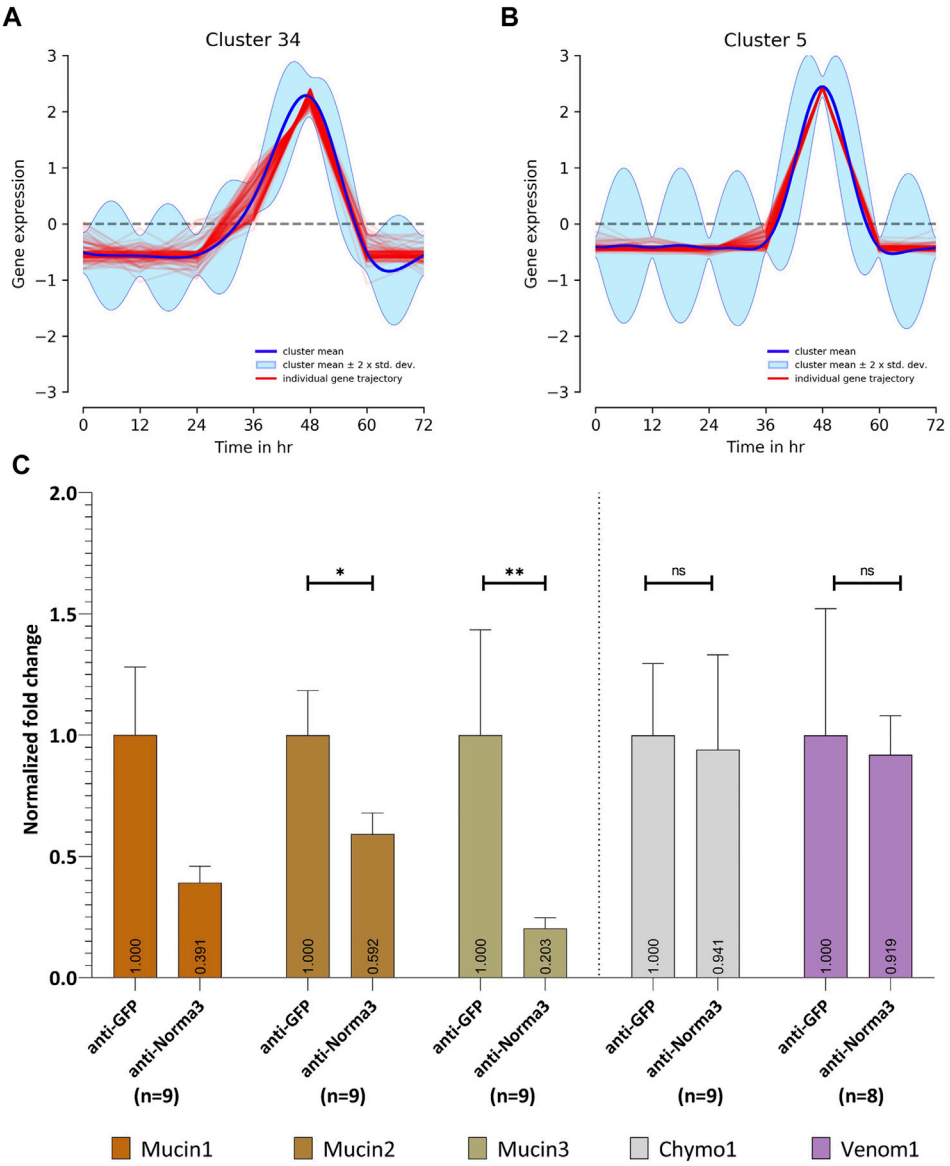

FIGURE 6
Expression patterns through Post-blood meal timepoints **(A,B)** and in relation to Norma3 silencing **(C)**. **(A)** Based on its expression across all timepoints, Norma3 was grouped in Cluster 34, which gradually peaks at 48 h Post-blood meal. **(B)** Three mucins neighboring Norma3 (mucin1-3) belong to Cluster 5 which presents an acute peak at 48 h. **(C)** Expression of Norma3-neighboring proteins in replicates of the anti-Norma3 vs. anti-GFP sample. All mucins exhibit a statistically significant expression drop. Mucin1 61% ($\bar{x}$ = 39.1 ± 19.3, n = 9), mucin2 41% ($\bar{x}$ = 59.2 ± 19.3, n = 9), mucin3 80% ($\bar{x}$ = 20 ± 12.5, n = 9) while other neighboring proteins (chymo1, venom1) do not (Mann-Whitney test). Both anti-GFP and anti-Norma3 samples contain 9 biological replicates. Results were normalized with ribosomal genes RpL32 and RpS17. All values are presented as mean ± SEM. *: p-value ≤0.05, **: p-value ≤0.01.

samples but initiated low transcription at 36 h and peaked at the end of vitellogenesis (around 48 h PBM) (Figure 6A). Among the 8 positively correlated neighbor transcripts, three mucins belonged to a cluster that presented an intense expression peak at 48 h (Figure 6B).

We then generated their detailed expression profile in NBF and PBM ovaries. We confirmed the expression pattern of five of the neighboring transcripts via qPCR. Three were annotated as mucin-2 and mucin-2-like (mucin1, 2, 3), one was annotated as cysteine-rich venom protein 6-like (venom1) and one as chymotrypsin inhibitor (chymo1). All five genes presented basal expression in NBF, 12, 24, 36, and 48 h ovaries. The expression of the three mucins presented a sharp increase between 12,000 (mucin2) and ~40,000-fold (mucin3) in 60 h

PBM ovaries, while chymo1 increased ~10,000-fold in 72 h PBM and venom1 3,000-fold in 60 h PBM ovaries (Supplementary Figure S3). To further assess the potential impact of Norma3 on the mucins we assayed the expression of the three mucins in a small sample of smaller ovaries (Figure 4A) of the anti-Norma3 60 h-PBM sample (that we collected as described in Section 3.4). Statistically significant downregulation between 40 to 80% of two out of the three mucin genes was detected in the smaller anti-Norma3 ovaries compared to anti-GFP control (Figure 6C). Downregulation of mucin3 presented the most significant effect, while no significant effect was detected on other protein-coding transcripts located in the same genomic region (mucin1, chymo1, venom1). Nevertheless, since we only tested nine smaller anti-Norma3 ovaries, we acknowledge that this result serves as a preliminary indication of the potential impact of Norma3 on mucin expression that should be validated with larger datasets.

4 Discussion

Long non-coding RNAs have arisen during the last decades as a fascinating field of research due to their unique features and intriguing mechanisms of action in the absence of a protein product. One entirely unexplored field of potential lncRNA applications is pest management and mosquito control. Due to the very low sequence conservation among lncRNAs (Diederichs, 2014; Tavares et al., 2019), such genes could be ideal as species-specific targets in new generation genetic control approaches. However, up to date there has not been sufficient research on specific lncRNAs to serve as efficient molecular targets with potential utility in pest management. In the tiger mosquito *Ae. albopictus*, a competent vector of multiple arboviruses, only a few studies regarding ncRNAs have been published so far (Gu et al., 2019; Azlan et al., 2021; Betting et al., 2021) and none presented significant data related to the impact of particular lncRNAs in physiological systems. Our aim was to provide the first proof-of-evidence regarding the role of specific lncRNAs in a vital biological process of the mosquito with potential utility for pest control. We focused on the reproductive system because of its significance for mosquito's viability and the broad applications that it may offer in mosquito control.

After computationally annotating predicted lncRNAs regarding their coding potential, genomic localization, species and developmental context specificity (Figure 1), we identified 10 species-specific lncRNAs which were overexpressed in *Ae. albopictus* ovaries upon blood-feeding (Figure 2A) and set out to further explore their potential role in reproduction. We deployed a loss-of-function RNAi-mediated pipeline and investigated the changes that occurred in the fecundity of female mosquitoes upon silencing of each lncRNA (Figure 2B). We focused on an antisense intergenic lncRNA, that we named Norma3, because its targeting provoked a robust phenotypic effect. Moreover, it exhibited an ideal expression profile: basal expression in most of the NBF/early PBM time points followed by a sharp increase by the end of vitellogenesis (Figure 3A). Its expression was also highly ovary-specific (Figure 3A), while its nucleotide sequence did not display any similarities to annotated genes of other species. It is worth mentioning that we observed a slight discordance between expression peaks of RNA seq data and qRT-PCR, probably due to different sampling methodologies or adaptation of the local *Ae. albopictus* strain to our laboratory conditions.

Our successful gene silencing approach (Figure 3B) resulted in significant reduction in oviposition and egg hatching (Figure 4C; Figure 5A) of the anti-Norma3 dsRNA-treated mosquitoes. We further associated this fertility reduction with defective ovaries (Figure 4B), smaller ovary follicle size (Figure 4A) and obvious malformations of 72-h embryos of the anti-Norma3 treated mosquitoes (Figure 5B). In addition, we attempted to obtain insights on the potential mode of action of Norma3. Long non-coding RNAs operate in a variety of different modes and uncovering their functional role is not a trivial task. Unlike protein-coding genes that share conserved domains due to sequence homology, the features of lncRNAs mainly arise from their secondary structures which perform complex interplay with DNA regions, RNAs or proteins (Pang et al., 2006; Ding et al., 2014). One of most frequent roles of lncRNAs is to regulate the expression of coding genes and, oftentimes, such genes are localized in the vicinity of a lncRNA (Rinn and Chang, 2012; Engreitz et al., 2016; Joung et al., 2017; Khyzha et al., 2019; Xing et al., 2021). To explore this possibility, we performed *in silico* analysis to highlight coding genes that possessed similar expression profiles as Norma3 and were localized in the same region ±100 kb up-or-down stream of Norma3 (Supplementary Data S2). Our search resulted in eight coding genes which were annotated as mucins, chymotrypsin inhibitors and cysteine-rich venom-like proteins, while all of them contained a trypsin inhibitor-like (TIL) cysteine rich domain. According to our qPCR results, the expression of two of these mucins was severely diminished upon injection of anti-Norma3 dsRNA (Figure 6C). While this evidence is circumstantial, it may indicate a possible interplay between Norma3 and the two neighboring mucins. Mucins are proteins that are characterized by domains that contain repetitive sequences of proline, threonine and serine (PTS domains) and heavy O-glycosylation (Tran and Ten Hagen, 2013) which are present in most metazoan (Lang et al., 2007). While mucins are the principal components of mucus and mucous membranes, they carry diverse roles from lubrication to cell signaling to forming chemical barriers.

To our knowledge, there are no direct studies associating mucins with oviposition in mosquitoes. Ovary-specific mucins have been highlighted in *Ae. albopictus* by a recent study that reported the upregulation of multiple mucins upon blood-feeding in females and speculated a potential role of these

genes in oviposition (Deng et al., 2020). Nonetheless, various ovary-specific mucins have been identified in other insects and studies have revealed their role in ovary development and reproduction. In particular, mucin-like proteins have been associated with the eggshell, a multilayered structure which is formed during oogenesis that protects and nurtures the developing embryo prior to its arrest (Osterfield et al., 2017). In *Drosophila melanogaster* three putative eggshell genes code for proteins with mucin-like domains (Muc4B, Muc11D, and Muc12Ea). Muc4B has been suggested to be a component of the wax layer of the embryo, while Muc11D and Muc12Ea potentially act as mediators of chorion hardening and coating for passage of the embryo through the oviduct (Tootle et al., 2011). Another ovary-specific mucin (NlESMuc) that was identified in plant grasshopper *Nilaparvata lugens* was also related to the eggshell and was proven essential for its fecundity. Specifically, the RNAi-mediated targeting of NlESMuc caused reduced oviposition, lower egg production and less egg hatching (Lou et al., 2019). Finally, a study in the lepidopteran *Spodoptera exigua* presented an ovary-specific mucin-like protein called Se-Mucin1 that was associated with choriogenesis. In the absence of Se-Mucin1, females exhibited reduced fecundity and the hatch rate of the eggs was also significantly impaired, while SEM analysis of the eggshell structures revealed that they were remarkably malformed (Ahmed et al., 2021).

Through our analysis we collected pieces of circumstantial evidence that indicates a possible interplay between Norma3 and two neighboring mucins (mucin 2, 3). Firstly, both RNA seq analysis (Figures 6A,B) and qPCR data (Figure 3A, Supplementary Figure S3) demonstrate tightly linked sharp expression increases of Norma3 and the three mucins at 60 h post-blood meal. Moreover, we detected a potential influence of anti-Norma3 treatment in the expression drop of mucins 2 & 3 that could be associated to the developmental delay of ovaries (Figure 6C). Anti-Norma3 treatment led to a downregulation of their expression, especially in mucin3 which exhibited the most intense and statistically significant effect (p-value: 0.0019). However, the current sample size of anti-Norma3 and anti-GFP ovaries should be enlarged in order to validate the finding. Given these preliminary results, we speculate that Norma3 may act as a positive regulator of the mucin cluster and its silencing could lead to inhibition of their ovary specific-expression and possibly to disruption of the reproductive ability of *Ae. albopictus*. *Cis*-acting lncRNAs are one of the most dominant lncRNA classes and the majority of them overlap with enhancers elements (Gil and Ulitsky, 2020). We also presume that Norma3 and the mucin proteins are involved in the formation of the eggshell, a hypothesis which is based on the well-studied role of mucins in other insects (Tootle et al., 2011; Osterfield et al., 2017; Lou et al., 2019; Ahmed et al., 2021), but also relies on the relevant phenotypic outcomes that were provoked by targeting an eggshell-related protein (EOF-1) in the relative species *Ae. aegypti* (Isoe et al., 2019). Further research is necessary to verify the hypotheses on both the impact of mucins on mosquito reproduction and their regulatory association with Norma3.

Our study presents a conceptual pipeline and a proof-of-principle towards novel approaches of insect pest control. It begins with the discovery of lncRNAs involved in the regulation of a physiological system that is fundamental for species survival and propagation, the reproductive system, and it showcases that down-regulating a particular lncRNA results in the damage of that system, reducing insect fecundity and fertility. Our results suggest that species-specificity of lncRNAs renders them preferred targets of RNAi-based pesticides. In fact, RNAi technology has offered a new and hopeful prospect of ecologically friendly approaches to insect control since it can minimize off-target effects. Low sequence conservation and high species-specificity of lncRNAs provide extra added value towards that end. Nonetheless, delivery methods of RNAi-based insecticides still pose major challenges (Yu et al., 2013; Niu et al., 2018) Till now, transgenic plants producing dsRNAs against vital insect genes has been the most straightforward and efficient application of RNAi technology for insect control (Nowara et al., 2010). However, this approach entails public acceptance problems due to the transgenic nature of the plants (Herman et al., 2021). Alternatively, spraying of stabilized dsRNA is recently being considered and actively researched [reviewed in (Rank and Koch, 2021)]. Examples come for the use of this technology against plant pests [e.g., sprays of dsRNA-producing *E. coli* against 28-spotted ladybird (Wu et al., 2021) or dsRNA sprays against Colorado potato beetle (Mehlhorn et al., 2020)], but one can easier envision house sprayings against biting mosquitoes due to greater dsRNA stability inside a house environment. Furthermore, reproductive system related lncRNAs, such as Norma3, could also be exploited in cutting-edge gene drive approaches aiming to suppress disease vectors by reducing female fertility (Hammond et al., 2016; Kyrou et al., 2018; North et al., 2020; Simoni et al., 2020). Given the available sequencing data in several insect species of public health or agricultural importance (or the affordability of obtaining such data from any organism of choice), this pipeline can be adopted to any given species and yield novel species-specific targets for pest control, thus addressing one of the most difficult challenges of the pesticide industry for species-specificity and environmental safety.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and

institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AB-T, M-EG, AG and KM conceived the study; AB-T and M-EG organized and designed the bioassays; AB-T. and OS performed the phenotypic and in vitro assays; AB-T conducted the statistical analysis; ST and AG analyzed RNA sequencing data; ST performed lncRNA annotation and clustering analysis; and AB-T and KM wrote the paper. All authors edited and reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.885767/full#supplementary-material

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Effects of antibiotics on the *in vitro* expression of tetracycline-off constructs and the performance of *Drosophila suzukii* female-killing strains

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Genetic control strategies such as the Release of Insects Carrying a Dominant Lethal (RIDL) gene and Transgenic Embryonic Sexing System (TESS) have been demonstrated in the laboratory and/or deployed in the field. These strategies are based on tetracycline-off (Tet-off) systems which are regulated by antibiotics such as Tet and doxycycline (Dox). Here, we generated several Tet-off constructs carrying a reporter gene cassette mediated by a 2A peptide. Different concentrations (0.1, 10, 100, 500, and 1,000 μg/mL) and types (Tet or Dox) of antibiotics were used to evaluate their effects on the expression of the Tet-off constructs in the *Drosophila* S2 cells. One or both of the two concentrations, 100 and 250 μg/mL, of Tet or Dox were used to check the influence on the performances of a *Drosophila suzukii* wild-type strain and female-killing (FK) strains employing TESS. Specifically, the Tet-off construct for these FK strains contains a *Drosophila suzukii nullo* promoter to regulate the *tetracycline transactivator* gene and a sex-specifically spliced pro-apoptotic gene hid^{Ala4} to eliminate females. The results suggested that the *in vitro* expression of the Tet-off constructs was controlled by antibiotics in a dose-dependent manner. ELISA experiments were carried out identifying Tet at 34.8 ng/g in adult females that fed on food supplemented with Tet at 100 μg/mL. However, such method did not detect Tet in the eggs produced by antibiotic-treated flies. Additionally, feeding Tet to the parents showed negative impact on the fly development but not the survival in the next generation. Importantly, we demonstrated that under certain antibiotic treatments females could survive in the FK strains with different transgene activities. For the strain V229_M4f1 which showed moderate transgene activity, feeding Dox to fathers or mothers suppressed the female lethality in the next generation and feeding Tet or Dox to mothers generated long-lived female survivors. For the strain V229_M8f2 which showed weak transgene activity, feeding Tet to mothers delayed the female lethality for one generation. Therefore, for genetic control strategies employing the Tet-off system, the parental and transgenerational effects of antibiotics on the engineered lethality and insect fitness must be carefully evaluated for a safe and efficient control program.

KEYWORDS

genetic control, transgenic sexing strain, tetracycline-off system, *Drosophila suzukii*, sterile insect technique, 2A peptide

Introduction

Genetic control strategies that introduce controllable sterility or lethality into the insect population have been developed in several insect species, including agricultural pests and human disease vectors (Harvey-Samuel et al., 2017; Alphey and Bonsall, 2018). Those strategies often mimic the principle of the sterile insect technique (SIT), which releases a large number of radiation-sterilized insects to suppress or eradicate the targeted pest population (Knipling, 1955; Klassen and Vreysen, 2021). Unlike classical SIT approaches that use wild-type strains and genetic sexing strains (GSSs) generated by classical genetics for mass-production, strategies such as Release of Insects Carrying a Dominant Lethal (RIDL) gene and Transgenic Embryonic Sexing System (TESS) took advantages of modern genetic engineering techniques. They generated insect strains carrying fluorescent gene marker(s) that could facilitate the monitoring of a release program and used lethal genes that kill insects at certain stages (Häcker et al., 2021). In addition, the tetracycline-off (Tet-off) system was used in those RIDL and TESS strains to control the expression of the lethal genes and allow population maintenance.

The conditionality of a Tet-off system is based on the interaction between the tetracycline transactivator (tTA) protein and the tetracycline response element (TRE), which can be intercepted by Tet or its derivatives such as doxycycline (Dox) (Gossen and Bujard, 1992; Berens and Hillen, 2003). When Tet is not present, tTA activates the expression of the TRE-linked effector gene, which was successfully demonstrated in *Drosophila melanogaster* (Bello et al., 1998). To introduce the controllable lethality, a cytotoxic or pro-apoptotic gene was linked to TRE to generate a Tet-repressible RIDL system in *Drosophila melanogaster* (Heinrich and Scott, 2000; Thomas et al., 2000). It was further discovered that *tTA* itself could be used as an effector gene to build an autoregulation loop, leading to the accumulation of tTA (Gong et al., 2005). Such overexpression of tTA typically killed insects at late developmental stages, such as late larvae or pupae, although the actual lethal mechanism behind it is still unknown (Phuc et al., 2007; Ant et al., 2012; Li et al., 2014; Concha et al., 2016). For TESS, promoters from cellularization genes were used to drive tTA, which activate the female-specific (fs) pro-apoptotic gene expression and kill all female insects at embryonic or early larval stages due to the induced apoptosis. The TESS strains were generated in tephritid fruit flies such as *Ceratitis capitata*, *Anastrepha suspensa*, and *A. ludens* (Schetelig and Handler, 2012b; Ogaugwu et al., 2013; Schetelig et al., 2016), as well as calliphorid blowflies such as *Lucilia cuprina* and *Cochliomyia hominivorax* (Yan and Scott, 2015; Yan et al., 2020b; Concha et al., 2020), which could reduce the rearing cost of a control program. Like TESS, female-specific RIDL can also be generated by using an fs-promoter for *tTA* or an fs-spliced intron for the effector gene (Thomas et al., 2000; Fu et al., 2007; Jin et al., 2013). Those female-killing (FK) strategies could lead to male-only releases, which are more effective than bi-sexual releases for an SIT program (Rendon et al., 2004; Schliekelman et al., 2005; Franz et al., 2021).

For an FK strategy using a Tet-off system, the regulator Tet plays a decisive role in the sexing efficiency and strain fitness. For example, the engineered insect lethality was directly linked to the concentrations of the Tet derivatives (Thomas et al., 2000; Horn and Wimmer, 2003). The lethal stages were also dependent on the timing of Tet removal (Gong et al., 2005; Schetelig et al., 2009; Yan and Scott, 2015), and the lethal phenotype can be reversed by adding Tet again (Horn and Wimmer, 2003; Schetelig et al., 2016). While low concentrations of Tet were not enough to suppress binding between tTA and TRE and the resulting lethality, high concentrations of Tet showed a deleterious effect on insect fitness (Schetelig et al., 2009; Schetelig and Handler, 2012a; Yan and Scott, 2015; Concha et al., 2020). We previously generated transgenic FK strains employing a Tet-off system in the spotted wing *Drosophila* (*Drosophila suzukii*; Diptera, Drosophilidae), a devastating fruit fly that recently became highly invasive (Asplen et al., 2015; Dos Santos et al., 2017). The Tet-off construct V229 that used to generate these FK strains contains an embryonic promoter from *Drosophila suzukii nullo* gene to regulate the *tTA* (driver cassette) and a sex-specifically spliced pro-apoptotic gene hid^{Ala4} (effector cassette) to eliminate female flies. While the most efficient FK strain eliminated all-female offspring at the embryonic stage if Tet was only fed to the larvae in the parental generation, some other strains killed the majority of females at late stages (Schetelig et al., 2021). It was speculated that Tet could be maternally inherited and switch off the embryonic lethality (Horn and Wimmer, 2003; Schetelig et al., 2009; Schetelig and Handler, 2012a; Yan et al., 2017). Here, we generated a series of Tet-off constructs that contain the same drive cassette as in V229 and a reporter gene cassette in which EGFP-NLS and DsRed-CAAX are co-expressed by a 2A peptide. By testing the Tet-off reporter constructs *in vitro* and *D. suzukii* WT or FK strains *in vivo*, we aim to investigate 1) how the antibiotics regulate the expression of Tet-off constructs at the cell level, 2) how the antibiotics affect the fly development and survival in the next generation, and 3) whether the antibiotics could suppress or delay the engineered lethality in our FK strains with different transgene activities.

Methods and materials

Insect rearing

The *Drosophila suzukii* wild-type (WT) USA strain and transgenic lines were maintained at 25°C and 55%–60% humidity under a 12L/12D photoperiod (Schwirz et al., 2020; Schetelig et al., 2021). The WT-USA strain was reared on an antibiotic-free diet, and the transgenic strains were kept on the same diet supplemented with 100 μg/mL Tet (Thermo Fisher Scientific).

Plasmid construction

Bicistronic gene cassettes containing EGFP-NLS (or DsRed-NLS) and DsRed-CAAX (or EGFP-CAAX), which were separated by a 2A peptide (DrosCV-2A or TaV-2A), were excised from V220_*pBXLII_attP_PUb-AmCyan_PUb-DsRed-NLS-DrosCV-2A-EGFP-CAAX-SV40*, V221_*pBXLII_attP_PUb-AmCyan_PUb-DsRed-NLS-TaV-2A-EGFP-CAAX-SV40*,V222_*pBXLII_attP_PUb-AmCyan_PUb-EGFP-NLS-DrosCV-2A-DsRed-CAAX-SV40*, or V223_*pBXLII_attP_PUb-AmCyan_PUb-EGFP-NLS-TaV-2A-DsRed-CAAX-SV40* (Schwirz et al., 2020) and used to replace the DsRed-NLS in the vector V206_*pXLBacII-attP-PUb-AmCyan_Dsnullo_DsRed-NLS-SV40* (Yan et al., 2020a) at the Bsu36I and MluI restriction sites, to generate V355_*pBXLII_attP_PUb-AmCyan_Dsnullo-DsRed-NLS-DrosCV-2A-EGFP-CAAX-SV40*, V356_*pBXLII_attP_PUb-AmCyan_Dsnullo-DsRed-NLS- TaV-2A-EGFP-CAAX-SV40*, V357_*pBXLII_attP_PUb-AmCyan_Dsnullo-EGFP-NLS-DrosCV-2A-DsRed-CAAX-SV40*, and V358_*pBXLII_attP_PUb-AmCyan_Dsnullo-EGFP-NLS-TaV-2A-DsRed-CAAX-SV40*, respectively. Similarly, the excised bicistronic gene cassette from V220, V221, V222, or V223 was used to replace the effector gene cassette $Dshid^{Ala4}$-*CctraF* in the vector

V229_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-Dshid*^{Ala4}-*CctraF-SV40* (Schetelig et al., 2021) at the Bsu36I and MluI restriction sites to generate V359_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-DsRed-NLS-DrosCV-2A-EGFP-CAAX-SV40*, V360_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-DsRed-NLS-TaV-2A-EGFP-CAAX-SV40*, V361_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-EGFP-NLS-DrosCV-2A-DsRed-CAAX-SV40*, and V362_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-EGFP-NLS-TaV-2A-DsRed-CAAX-SV40*, respectively.

Cell culture experiments

The cell transfection and counting were carried out as previously described (Yan et al., 2020a; Schetelig et al., 2021). Briefly, *Drosophila* Schneider 2 (S2) cells were grown on Schneider's medium containing 10% heat-inactivated fetal bovine serum (Hi-FBS) and 1% penicillin/streptomycin in closed-capped flasks without CO_2 at 25°C. The Xfectin Transfection Reagent (Takara), 1 μg of plasmid DNA, and Tet or Dox (Alfa Aesar) at 0.1, 10, 100, 500, or 1,000 μg/mL (correspondingly named Tet-0.1, Tet-10, Tet-100, Tet-500, Tet-1000, Dox-0.1, Dox-10, Dox-100, Dox-500, and Dox-1000) were used for transient transfections. A 13-mm TC coverslip (Sarstedt) was placed into each well of a 24-well plate to facilitate imaging. The transfection was stopped by refreshing the dishes with 500 μL of Schneider's medium containing 10% Hi-FBS and 1% penicillin/streptomycin. The cells were incubated for ~18 h at 25° C before fixing in 4% paraformaldehyde for 15 min and washing briefly with PBS prior to microscopy. The transfected cells were imaged using a M205FA microscope (Leica Microsystems) with filter sets CFP for AmCyan (ex. 436/20; em. 480/40), YFP for EGFP (ex. 510/20; em. 560/40), TxRed for DsRed (ex. 545/30; em. 620/60), and GFP-LP for overlay (ex. 425/60; em. 480). Fluorescent cells were counted in ImageJ (Fiji) using the automated cell count function. The raw images were converted to an 8-bit standardized format (threshold 30) and inverted before a watershed was applied to separate any cells in direct contact. The ratio of green or red to blue cells was calculated. To evaluate the effects of antibiotic treatments on cell viability, cells were grown with Tet or Dox at a certain concentration as mentioned previously without plasmid DNA. After incubation, the live cells were counted using a TC20 automated cell counter (Bio-Rad).

ELISA (enzyme-linked immunosorbent assay) experiment

The antibiotic-supplemented diets were fed to the flies at both larval and adult stages (L+/A+), or only at the larval stage (L+/A−). For the latter, the pupae were collected and rinsed with ddH_2O twice before transferring to antibiotic-free vials. To test the maternal or paternal effects, the newly emerged males and females were crossed with their WT partners for 1–2 weeks. The F1 generation was reared on the antibiotic-free diet. Then, 100 eggs (0–6 h collection) and 10 adult females (1 day old) from each cross were collected as test samples for ELISA (Elabscience Biotechnology). In addition, 10 WT adult females (1 day old) from antibiotic-free and Tet-100-containing diets were collected as negative and positive controls, respectively. Three biological replicates were used for each cross and control. A measure of 400 μL of trichloroacetic acid solution (1%) and beads (Lysing Matrix D Bulk, Cat. 6540-434, MPbio, France) were added to the sample tube, and the tissues were homogenized using a Precellys 24 homogenizer (for 20 s at 6000 rpm). The sample tubes were centrifuged at 4,000 g for 10 min at room temperature. Then, the supernatant (27.5 μL) was mixed with reconstitution buffer (82.5 μL), and 50 μL of the mixture was used for analysis. The standard solution (1.0 ppm) was diluted according to the manufacturer's instructions. A measure of 50 μL of diluted standard solution and sample mixture was added per well of the pre-coated 96-well microtiter plate in duplicate; 50 μL of the antibody working solution was added to each well, and the plate was covered with a lid, gently oscillated for 5 s, and incubated with shading light at 37°C for 30 min. After incubation, the microplate wells were washed six times with 250 μL/well of washing buffer, 100 μL/well of streptavidin–horseradish peroxidase (HRP conjugate) was added, and the plate was incubated at 37°C for 30 min in the dark. Then, the plate was washed again as described previously, and 50 μL of each substrate reagent A and B was added sequentially per well. After incubation at 37°C in the dark for 15 min, the reaction was stopped by adding 50 μL/well of stop solution. Optical density (OD) at 450 nm and 630 nm of each well was measured as reference wavelength (TECAN microplate reader). The concentrations (ppb) were calculated as the OD value measured at 630 nm subtracted from that measured at 450 nm. The absorbance percentage was calculated using the following formula: absorbance (%) = A/A0 × 100% (A: average absorbance of the standard solution or sample; A0: average absorbance of 0 ppb standard solution). The average absorbance value from duplicate wells was added to the standard curve. The concentration calculated from the standard curve was multiplied by 8 (the dilution factor for the pretreatment of tissue/egg samples according to the manual) for the final concentration of the samples.

Biological experiments

To evaluate the effect of antibiotic treatments on fly survival and development time (from embryo to adult), the diet supplemented with the indicated antibiotics was fed to WT-USA flies, and adults were crossed as previously described. A total of 100 eggs were collected and dechorionated before transferring them to antibiotic-free vials. Dechorionation was conducted by submerging eggs into 50% bleach solution (Dan Klorix, Colgate-Palmolive, Hamburg, Germany) for 3.5 min. Then, the eggs were rinsed three times with distilled water to remove potential bleach residues. The number and the development time (days) of the resulting males and females were recorded. Eggs without dechorionation were tested in a similar manner as the control. To test the antibiotic treatments on the female lethality of FK strains, the diet with Tet-100 or Dox-100 was fed to the V229_M4f1 heterozygous larvae. The pupae were collected as described previously, and the newly emerged males and females (<4 h) were kept separately in the antibiotic-free vials for 1, 5, or 10 days before they were crossed with WT partners. This allowed different periods for antibiotic degradation in the flies. The flies were transferred daily to a fresh vial with a tetracycline-free diet for 9 days (10 vials in total). The fluorescent and non-fluorescent F1 adults (<24 h) from these 10 vials were sexed and counted. Meanwhile, the 1-day-old V229_M4f1 flies were crossed, and the offspring were reared and measured similarly, except that food with

Tet-100 or Dox-100 was used throughout the experiments as controls. To assess the female survival, the V229_M4f1 larvae were fed with a diet containing Tet-100, Dox-100, or Dox-250, and the F0 adults were crossed on an antibiotic-free diet as described previously. The F1 transgenic females were collected every day in separated vials until no more females emerged. The females in those vials were counted every 2 days before transferring to a new vial (antibiotics-free) until all flies died. Meanwhile, the newly emerged F1 transgenic and WT males were counted to evaluate the male production under different antibiotic conditions. To verify the transgenerational effect of antibiotics, the diet with Tet-100 was fed to the V229_M8f2 heterozygous larvae, and the F0 and F1 transgenic females were crossed with WT males as described previously. The newly emerged F1 or F2 transgenic flies were sexed and separated into antibiotic-free food vials (20 flies per vial). These flies were counted every 2 days before transferring to new vials until 40 days or all flies died. The WT flies from antibiotic-free diet were counted similarly as the control. Here, three biological replicates were carried out for all the experiments.

Statistics

Statistical analysis was carried out using SigmaPlot (v14.0). Differences in the green/blue or red/blue cell ratios, fly development or survival from different treatments, antibiotic concentrations (ELISA assays), or male production from different crosses were analyzed by one-way analysis of variance (ANOVA), and means (some are square root-transformed) were separated using either the Student–Newman–Keuls, Holm–Šidák, or Duncan's method. Differences in the female lethality between the WT and transgenic offspring from different antibiotic-treated mothers or fathers were analyzed by the paired *t*-test. Differences in the survival rates of female offspring from antibiotic-treated mothers and fathers were analyzed using the z-test.

Results and discussion

Tet and Dox regulated the expression of Tet-off constructs *in vitro*

We previously characterized promoters from four *Drosophila suzukii* cellularization genes, including *nullo*, *serendipity-α* (*sry-α*), *bottleneck* (*bnk*), and *slow-as-molasses* (*slam*). The *Dsnullo* promoter regulated the strongest *DsRed-NLS* gene expression in *Drosophila melanogaster* S2 cells (Yan et al., 2020a). We also previously generated *piggyBac* vectors containing a bicistronic gene cassette in which EGFP-NLS (or DsRed-NLS) and DsRed-CAAX (or EGFP-CAAX) were co-expressed by a 2A peptide (DrosCV-2A or TaV-2A) (Schwirz et al., 2020). The bicistronic gene cassette was regulated by the *Drosophila melanogaster polyubiquitin* (*DmPUb*) promoter, and *Drosophila suzukii* transformed by these *piggyBac* vectors showed whole-body green and red fluorescence (Schwirz et al., 2020). Here, we generated a range of *piggyBac* constructs in which the bicistronic gene cassette was either under the direct control of the *Dsnullo* promoter (V355–V358) or regulated by the TRE element that responds to *Dsnullo*-controlled tTA (V359–V362) (Figure 1A).

Drosophila S2 cells transfected with V355–V358 showed weak green fluorescence and no red fluorescence, regardless of the position of EGFP-NLS or DsRed-NLS to the 2A peptide (Figure 1B). Those cells also showed bright blue fluorescence driven by the *DmPUb* promoter (Figure 1B). The undetectable red fluorescence is possibly due to the relatively weak activity of the *Dsnullo* promoter compared to that of the *DmPUb* promoter and longer maturation time of the DsRed.T3 protein than EGFP (Bevis and Glick, 2002). Earlier *in vitro* studies showed that the combination of the tTA driver and TRE lethal effector components, but not a single component, mediated efficient cell death when antibiotics were absent (Schetelig and Handler, 2012a; Schetelig et al., 2021). Similarly, cells transfected with V360 or V362 (employing TaV-2A) showed intense green and red fluorescence (Figure 1B). This suggests a strong expression induction of the bicistronic gene cassette triggered by the binding of *Dsnullo*-regulated tTA to the TRE and efficient protein translation mediated by TaV-2A. On the other hand, cells transfected using V359 and V361 (employing DrosCV-2A) showed weak or moderate expression of EGFP-NLS and DsRed-NLS (Figure 1B). This suggested that the cleavage activity of DrosCV-2A is lower than that of TaV-2A when the bicistronic cassette is placed after TRE. Both DrosCV-2A and TaV-2A have been previously used to co-express two different pro-apoptotic genes including *reaper*, *head involution defective* (*hid*), and *grim*. S2 cell death assays showed that the lethality can be significantly enhanced by such binary expression compared to the single expression of a pro-apoptotic gene (Jaffri et al., 2020). Here, our observation suggested that TaV-2A may be preferred over DrosCV-2A for multiple expression of different lethal genes using the Tet-off system.

The weak expression of the reporter genes in V359 (DsRed-NLS_DrosCV-2A_ EGFP-CAAX) is possibly due to the position of DsRed relative to DrosCV-2A. It was found that translation was less efficient for DsRed when placed upstream of the 2A peptide than downstream (Wang et al., 2019). In addition, the cell imaging for V361 and V362 showed the loss of membrane integrity (Figure 1B), suggesting that DsRed aggregates disrupt cell walls. We previously identified a detrimental effect on cells *in vitro* and *in vivo* when overexpressing the DsRed.T3 with the membrane tag CAAX (Schwirz et al., 2020). Specifically, transgenic *Drosophila suzukii* expressing DsRed-CAAX showed extensive membrane blebbing and are homozygous lethal. Therefore, vectors containing DsRed-CAAX could be used to generate conditional lethal strains.

Since V360 and V362 exhibited strong expression of the reporter genes when no antibiotic was present, they were chosen to analyze the suppression of these genes in response to Tet or Dox treatments (Figure 1C). The V222 and V356, in which the bicistronic cassette is regulated by *DmPUb* and *Dsnullo* promoters, respectively, were also used as controls since they do not contain Tet-off components and thus should not respond to the antibiotic treatments. Since all tested constructs have a *DmPUb-AmCyan* cassette, the cells showing blue fluorescence suggested that these cells were transfected successfully. The number of red or green cells was then related to the number of blue cells to show the relative expression of DsRed or EGFP, which were controlled either by the *Dsnullo* promoter or TRE. When using V222 with no antibiotics, Tet-100 or Dox-100 for transfection, green and red cell ratios (related to blue cells) were in the range of 54.1%–66.4% and 23.4%–25.3%, respectively. The ratios for green or red cells under these treatments were not significantly different ($p > 0.05$, one-way ANOVA). Similarly, for V356, the green and red cells ratios were

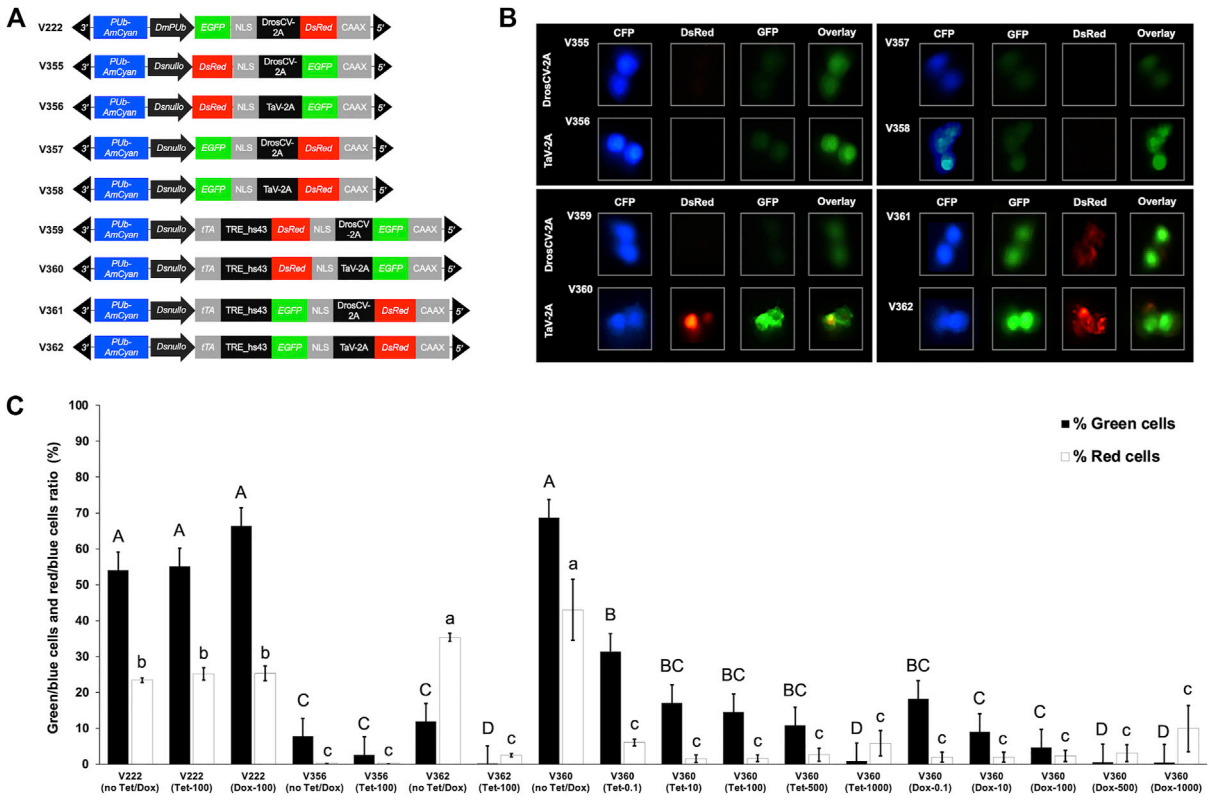

FIGURE 1
Effects of antibiotics on the expression of tetracycline-off (Tet-off) constructs in *Drosophila* S2 cells. **(A)** Schematic map of the *piggyBac* vectors containing a bicistronic gene cassette for EGFP-NLS (or DsRed-NLS) and DsRed-CAAX (or EGFP-CAAX), separated by a 2A peptide, expressed under the control of either the *D. melanogaster* PUb promoter (DmPUb), the *D. suzukii nullo* promoter (Dsnullo), or a TRE regulatory element that responds to Dsnullo-controlled tTA. Constructs contained DmPUb-AmCyan as an independent visible marker. **(B)** *Drosophila* S2 cells not treated with antibiotics and transfected with *piggyBac* vectors are shown. Images were taken under epifluorescent light conditions. **(C)** Ratios of green and red fluorescent cells relative to blue fluorescent cells are depicted, and the respective antibiotic treatments are indicated. Fluorescent cells were detected and counted using ImageJ, and the ratio of green/blue or red/blue cells was calculated. Each bar represents the mean ± SE of $n = 3$ experiments. Letters describing significant differences at $p < 0.05$ (one-way ANOVA or Student–Newman–Keuls method) are given for green (upper case) and red cell measurements (lower case).

in the range of 2.6%–7.8% and 0.0%–0.1%, respectively, with no significant differences in these treatments ($p > 0.05$, one-way ANOVA). Therefore, the expression levels of the reporter genes in V222 and V356 were independent of the antibiotic treatments. On the other hand, the ratios of green and red cells for V362 were 11.9% and 35.3%, respectively, when the antibiotic was absent. Those ratios were significantly reduced to 0.1% and 2.5% ($p < 0.05$, one-way ANOVA), respectively, when Tet-100 was present. This indicated that for V362 the Tet at 100 μg/mL concentration effectively intercepted the binding between tTA and TRE, thereby suppressing the reporter gene expression. Since the DsRed-CAAX from V362 disrupted the integrity of the cell walls (Figure 1B), it is possible that cells were killed by such a detrimental effect and not counted in the assay.

Due to the detrimental effect of DsRed-CAAX on cells and high cleavage activity of TaV-2A, V360 (DsRed-NLS_TaV-2A_EGFP-CAAX) was used for further dose-dependent experiments. Without plasmid DNA, treatments with no antibiotics, Tet-0.1, Tet-10, Tet-100, Tet-500, Dox-0.1, Dox-1, and Dox-10 generated >70% alive cells, whereas treatments with Tet-1000, Dox-100, Dox-500, and Dox-1000 reduced the alive cells to 47%, 60%, 33%, and 27%, respectively (Supplementary File S2). This suggested that high doses of antibiotics reduce the cell viability. Alive cells transfected with V360 from different antibiotics treatments were further imaged and counted. The ratio of green/blue cells for V360 was 68.8% when no antibiotics were present (Figure 1C), similar to the corresponding ratio for V222. Adding Tet-0.1, Tet-10, Tet-100, or Tet-500 or Dox-0.1 significantly reduced the ratio of green/blue cells to 10.9%–31.4% ($p < 0.05$, one-way ANOVA). There was no significant difference among the green/blue cell ratios under these treatments ($p > 0.05$, one-way ANOVA). Adding Tet-1000, Dox-10, Dox-100, Dox-500, or Dox-1000 further reduced the ratio of green/blue cells to 0.5%–9.0%, significantly lower than that from the Tet 0.1 treatment ($p < 0.05$, one-way ANOVA). Meanwhile, the ratio of the red/blue cells was 43.0% when no antibiotic was present, significantly higher than that from V222 ($p < 0.05$, one-way ANOVA). Adding Tet or Dox at all tested concentrations significantly reduced the red/blue cell ratios to 1.5%–9.9% ($p < 0.05$, one-way ANOVA). There was no significant difference in the red/blue cell ratios under these treatments ($p > 0.05$, one-way ANOVA). Likely, the cell ratios from high-dose treatments such as Tet-1000, Dox-100, Dox-500, and Dox-1000 do not show the overall expression of reporter cassettes since fewer cells were viable compared to other

treatments (Supplementary File S2). This may explain why these high-dose treatments did not further reduce the expression in a significant manner.

Since the translation efficiency may be hindered by placing DsRed before the 2A peptide and the DsRed protein needs a longer maturation time than EGFP (Bevis and Glick, 2002; Wang et al., 2019), the ratio of green/blue cells might be a better reference for the expression level of the bicistronic cassette than red cell counting. The results from green/blue cell ratios *in vitro* assays collectively suggested the following: 1) the expression of Tet-off constructs was sensitive to antibiotic treatments since low Tet or Dox levels (0.1 μg/mL) effectively reduced the fluorescent cells; 2) the high levels of Tet or Dox (500 or 1,000 μg/mL) were required to get the maximal suppression of reporter gene expression; 3) Dox exhibited overall higher efficiency than Tet in suppressing the reporter gene expression; 4) while the *Dsnullo* promoter itself mediated low reporter gene activity (indicated by the low ratio of green/blue cells from V356), the Tet-off system using the same promoter for tTA increased the reporter gene expression to a level similar to using a strong *DmPUb* promoter (ratio of green/blue cells from V222).

Feeding Tet to *Drosophila suzukii* WT flies showed different effects on the development and survival of their offspring

Tet at 100 μg/mL (Tet-100) was commonly used to rear the insect strains in which lethality is controlled by Tet-off systems (Gong et al., 2005; Schetelig et al., 2009; Ant et al., 2012; Schetelig and Handler, 2012a; Schetelig and Handler, 2012b; Jin et al., 2013; Yan and Scott, 2015), and Tet at 10 μg/mL (Tet-10) or lower was shown to be sufficient to maintain lethal strains of *Drosophila melanogaster* and *C. capitata* (Horn and Wimmer, 2003; Schetelig et al., 2009; Li et al., 2014; Upadhyay et al., 2022). In an SIT-like control program using transgenic sexing strains based on Tet-off systems, the last generation before release will be switched to non-Tet diet to produce male-only populations. For the sexing strains engineered with late lethality, Tet is often supplied to the adults, and rearing the offspring without Tet would trigger the designed lethality (Heinrich and Scott, 2000; Thomas et al., 2000; Fu et al., 2007). For the strains engineered with embryonic lethality, Tet should only be supplied to larvae but not adults to minimize the parental Tet that could suppress the early lethality in the offspring (Schetelig and Handler, 2012a; Schetelig and Handler, 2012b; Yan and Scott, 2015). Here, the impact of parental Tet on the fitness of the offspring using our WT-USA strain was tested. To avoid any Tet that might be passed on from the parental flies, the eggs collected from different treatments were dechorionized using a bleach-based method. By applying non-antibiotic conditions and dechorionation (L−/A−_decho), the development time (from egg to adult) of the female and male offspring was 16.1 and 16.5 days, respectively, which were comparable to those (17.7 and 16.8 days for female and male offspring, respectively) from non-antibiotic conditions, but without dechorionation (L−/A−_intact). Compared to the "L−/A−" treatment, feeding Tet-10 at larval and adult stages (Tet-10_L+/A+), feeding Tet-100 at only the larval stage (Tet-100_L+/A−), feeding Tet-100 at larval and adult stages (Tet-100_L+/A+), and feeding Tet-100 only to males (Tet-100_L+/A+_M) or females (Tet-100_L+/A+_F) delayed the developmental time of females to 22.2, 21.7, 20.2, 23.9, and 21.7 days, respectively ($p < 0.001$, one-way ANOVA; Figure 2A). Similarly, these treatments delayed the developmental time of male offspring to 22.7, 20.1, 20.3, 24.4, and 22.1 days, respectively ($p < 0.001$, one-way ANOVA). On the other hand, there was no significant difference in the survival of female or male offspring from treatments involving F0 sibling crossing, such as "L−/A−_intact," "L−/A−_decho," "Tet-10_L+/A+," "Tet-100_L+/A−," and "Tet-100_L+/A+," despite the antibiotic treatments ($p > 0.05$, one-way ANOVA; Figure 2B). However, treatments involving inter-population crossing, including "Tet-100_L+/A+_M" and "Tet-100_L+/A+_F," significantly reduced the number of their offspring ($p < 0.05$, one-way ANOVA; Figure 2B).

The development and survival (from egg to adult) of *Drosophila suzukii* is largely determined by variables for rearing such as temperature, humidity, and diet (Winkler et al., 2020). A summary from multiple studies showed that at temperatures close to 24 or 25°C, but different humidity and diet, the developmental times were in the range of 10–13 days and the survival rate were at 55%–85% (Winkler et al., 2020). Under our non-antibiotic conditions, the developmental time was at 16–17 days and thus longer compared to Winkler et al., possibly due to the humidity, diet and/or handling differences. Nevertheless, feeding Tet to the parents or only to the parental males or females delayed the development time of the offspring for 3.6–7.9 days (Figure 2A). This suggested some parental effects induced by the Tet treatment in the adults and/or an effect of the maternal Tet. To detect the presence of antibiotics in the treated flies and their offspring, ELISA was performed based on a Tet antibody. The assay detected 34.8 ng/g Tet in adult females that fed on Tet-100 at both larval and adult stages (Supplementary Figure S1). However, the amount of antibiotics in the eggs from different treatments, including different antibiotics (Tet or Dox), different concentrations (10, 100, or 250 μg/mL), different treated stages (larvae or adults), and treating males or females, were calculated and compared to non-antibiotic controls (Supplementary Figure S1; $p > 0.05$, one-way ANOVA). Such levels (0.5–3.4 ng/g) are also below or close to the detection limit (2.4 ng/g) of the ELISA kit. The results indicated that either antibiotics were not deposited into the eggs by their parents, or the ELISA kit is not suitable to detect antibiotics in *Drosophila* eggs.

While all treatments involving antibiotics delayed the developmental time in the next generation (Figure 2A), most of them did not affect the fly survival (Figure 2B). For a control program of *D. suzukii* using insect strains carrying Tet-off systems, these results would mean that the insect production in the pre-release generation should not be negatively affected by the Tet diet that was fed to the parental generation. Interestingly, crossing Tet-treated flies with their untreated partners resulted in a severe reduction in the offspring number (Figure 2B). Since feeding Tet to both parents should have stronger effects (if not the same) on the performance of their offspring compared to only feeding Tet to one parent, it is unlikely that the Tet treatment to father or mother caused the reduction in the offspring number. *Drosophila* species are known for assortative mating, which describes the phenomena that individuals mate assortatively with others of similar environmentally induced phenotypes (Robinson et al., 2012; Najarro et al., 2015). We also observed strong assortative mating among different *D. suzukii* populations in our laboratory (data not shown). Such assortative mating may promote the sexual reluctance between individuals from antibiotic-treated and -untreated populations. It is possible that some eggs collected from such crosses were not fertilized (even if the flies were crossed for >1 week) and therefore never hatch. On the other hand, for all

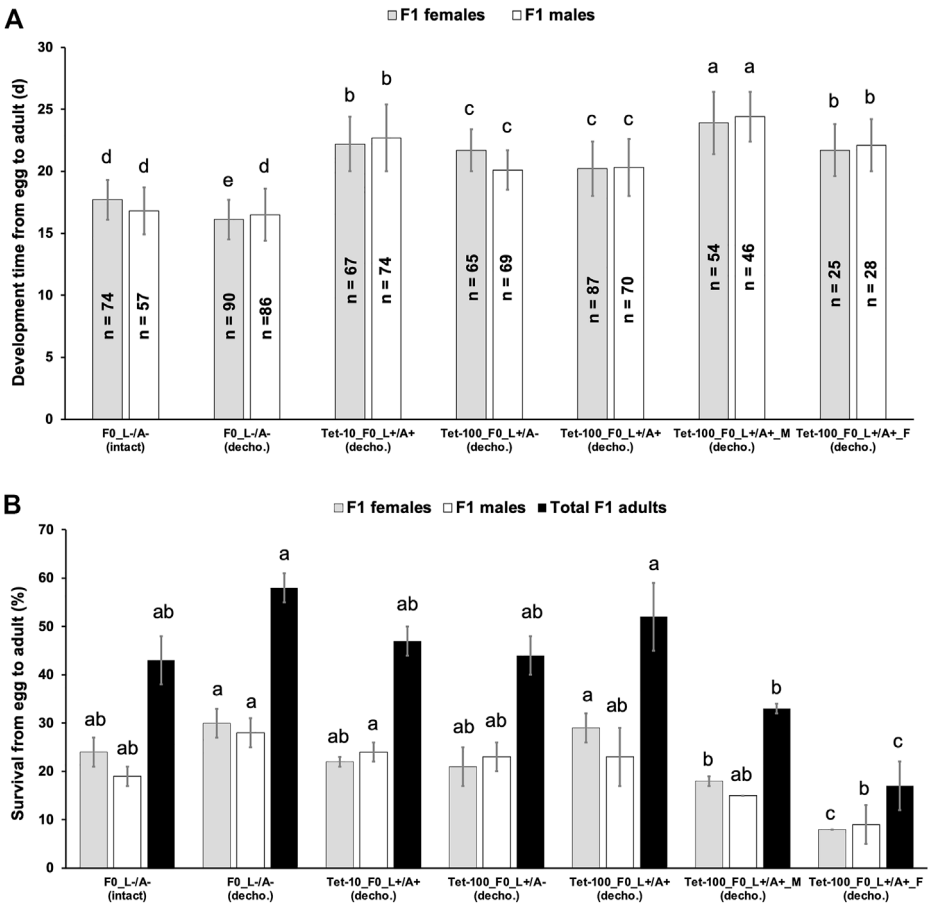

FIGURE 2
Effects of parental antibiotics on the development time **(A)** and survival **(B)** of the *D. suzukii* WT-USA strain. The flies from the parental generation (F0) were fed with food containing 10 or 100 μg/mL tetracycline (Tet-10 or Tet-100) at both larval and adult (L+/A+) stages or only at the larval stage (L+/A–) or were always fed with food containing no antibiotics (L–/A–). Then, 100 dechorionized (decho.) or intact eggs were transferred to an antibiotic-free vial, and the developmental time (days) from egg to adulthood as well as the number of newly emerged F1 flies was recorded. n represents the tested individuals. The mean and standard error from three replicates are shown. Different lower-case letters above the bars from the same kind of F0 flies indicate significant difference ($p < 0.05$, one-way ANOVA).

treatments which showed no impact on the offspring survival, siblings from the same population were used for the crosses, and thus females were more likely to mate. The assortative mating was reported as an obstacle for the SIT application against the melon fly (Hibino and Iwahashi, 1991; Koyama et al., 2004). Therefore, the assortative mating between the mass-rearing population using antibiotic-supplemented diets and the targeted wild *D. suzukii* populations with no or limited contact to antibiotics should be investigated for the implementation of any genetic control strategies that are based on antibiotic-fed systems. One useful tool for such purpose is the sperm-marking strain that can be employed to monitor the (assortative) mating among certain populations (Ahmed et al., 2019; Yan et al., 2021).

Feeding Dox to F0 flies inhibits the F1 female lethality in the *D. suzukii* FK strain V229_M4f1

In the *in vitro* study using Tet-off constructs, applying high levels of Tet or Dox (500 or 1, 000 μg/mL) could minimize the effector gene expression (Figure 1C). However, such treatments had severe negative impact on the cell viability (Supplementary File S2) as well as the fitness of *D. suzukii* flies *in vivo* (Schetelig et al., 2021). Therefore, they may not be considered for practical use. Earlier studies suggested that the amount of Tet needed to suppress the Tet-off system-mediated lethality was subjected to insect species and strains with certain transgene activity. While Tet at 100 μg/mL was commonly used as previously mentioned, in other species and Tet-off systems, higher concentrations up to 200 μg/mL were needed to completely suppress the lethality (Tan et al., 2013; Concha et al., 2020). At even higher concentrations (300 μg/mL), the insect fitness of *C. capitata* and *Anastrepha suspensa* was severely affected (Schetelig et al., 2009; Schetelig and Handler, 2012a). Therefore, we selected 100 and 250 μg/mL as test concentrations for most experiments. The *Drosophila suzukii* FK strain V229_M4f1 was previously generated by germline transformation using the vector V229*_pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-Dshid*^{Ala4}*-CctraF-SV40* (Schetelig et al., 2021). The V229_M4f1 strain showed moderate lethality and killed females mostly at the pupal and adult stages when Tet was not present. Here, we fed Tet-100 or Dox-100 to V229_M4f1 larvae, separated the newly emerged males and females, and crossed them at different ages with their WT partners on non-antibiotic food.

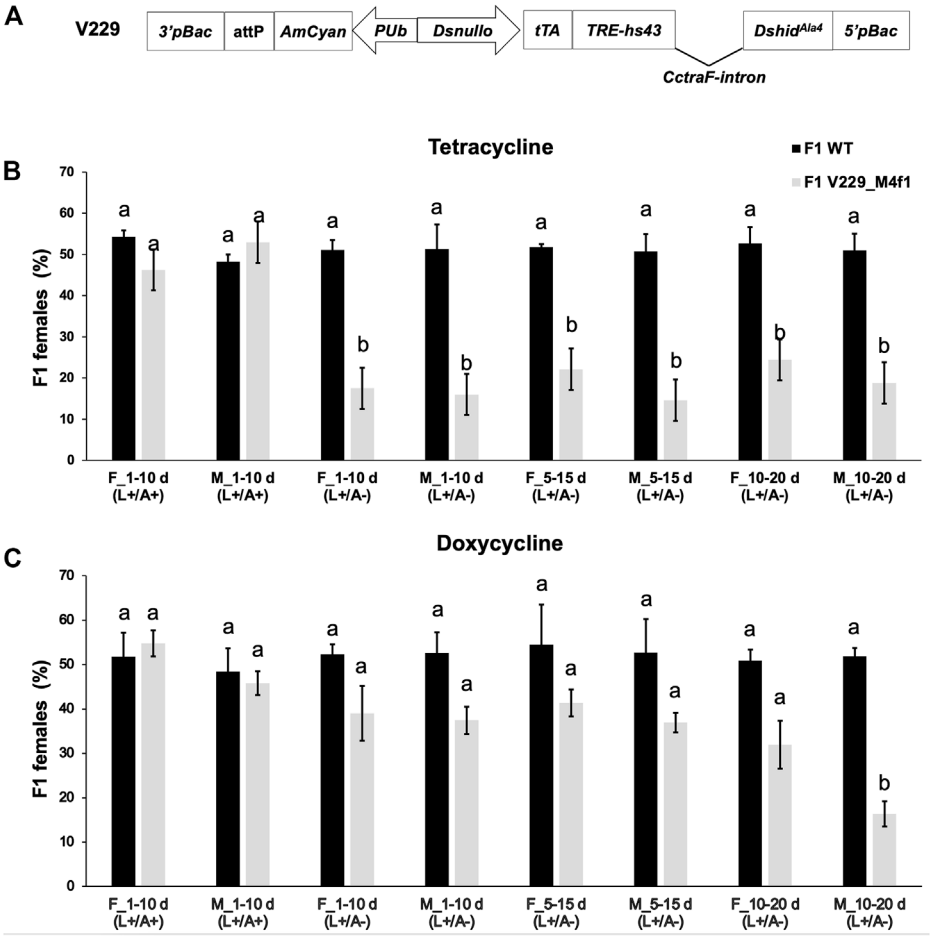

FIGURE 3
Parental effect of antibiotics on the female lethality of the *Drosophila suzukii* female-killing strain V229_M4f1. **(A)** V229_M4f1 was previously generated by the germline transformation using the vector V229_*pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-Dshid*^{Ala4}*-CctraF-SV40* which contains the female-specific *transformer* intron from *C. capitata* (*CctraF*), as well as an AmCyan marker gene regulated by the constitutive *D. melanogaster polyubiquitin* (*PUb*) promoter and an *att*P recombination site (Schetelig et al., 2021). Transgenic larvae (heterozygous) from the parental generation (F0) were fed on diets containing 100 μg/mL tetracycline **(B)** or doxycycline **(C)**. The newly emerged males (M) and females (F) were separated into different antibiotic-free vials, and 1-, 5-, or 10-day (d)-old males and females were crossed with their WT partners (4–7 days old) on antibiotic-free diet. Crosses were maintained for 10 days by transferring the flies to a fresh vial (antibiotic-free diet) every day. The fluorescent and non-fluorescent (WT) F1 adults from these ten vials were sexed and counted. Similarly, control experiments were carried out using 1-day-old transgenic flies and antibiotic-treated diets. +/−, antibiotic-treated diet (100 μg/mL) used for F0 larvae before switching to an antibiotic-free diet for F0 and F1 flies; +/+, antibiotic-treated diet (100 μg/mL) used for all F0 and F1 stages. The mean and standard errors from three replicates are shown. Letters above the bars indicate significant differences ($p < 0.05$, paired *t*-test).

Previously, antibiotic degradation was observed in the RIDL strain (OX513A) of the yellow fever mosquito *Aedes aegypti* (Curtis et al., 2015), possibly due to the insect metabolism or the binding of large amounts of Tet by high concentrations of the tTA protein produced in RIDL systems. Therefore, flies crossed at an older age were expected to pass lower amounts of antibiotics to their offspring due to degradation. Since heterozygous V229_M4f1 flies were crossed, half of the progeny were WT with a 1:1 sex ratio (Figure 3), while the other half were transgenic flies (heterozygous) with a sex ratio subject to the parental antibiotics. Previously, crossing V229_M4f1 males with WT females on non-Tet diet produced transgenic offspring consisting of only 14.2% females (Schetelig et al., 2021). When Tet-100 was used here, regardless of the F0 sex and age, the female percentages of transgenic adults were in the range of 14.6%–24.5%, which were significantly lower than those of WT in each cross ($p < 0.05$, paired *t*-test) (Figure 3A). Therefore, feeding Tet-100 to F0 flies did not rescue the female lethality in F1. When using Dox-100, most of the female percentages of transgenic flies were in the range of 31.9%–41.3%, not significantly different from those of WT flies ($p > 0.05$, paired *t*-test) (Figure 3B). Such observation suggested that the F1 female lethality was suppressed, possibly due to some Dox-induced parental effects and/or an effect of the inherited Dox. The only exception was the cross using 10–20-day-old V229_M4f1 males, which again produced transgenic offspring with a strong male-biased sex ratio (the female percentage was 16.4%), indicating that either Dox degraded faster in males or males contributed less amount of Dox to the offspring than females at this age. When both F0 and F1 generations were maintained on Tet or Dox diet, no female lethality was observed in the transgenic F1 flies.

TABLE 1 Effects of parental antibiotics on the female survival rates in the *D. suzukii* FK strain V229_M4f1.

Antibiotic[a] concentration	F0 flies[b]	F1 female survival (%)[c]				
		Day 2	Day 4	Day 6	Day 8	Day 10
Tet-100	Female	13.8 a (22/160)	3.7 a (6/160)	1.9 a (3/160)	1.3 (2/160)	0.6 (1/160)[d]
	Male	5.0 b (11/218)	0.4 b (1/218)	0 a (0/218)	—	—
Dox-100	Female	21.4 a (22/103)	5.8 a (6/103)	3.9 (4/103)	1.0 (1/103)	0 (0/103)
	Male	2.1 b (3/143)	0 b (0/143)	—	—	—
Dox-250	Female	25.8 a (18/70)	4.3 (3/70)	1.4 (1/70)	1.4 (1/70)	1.4 (1/70)[e]
	Male	0 b (0/94)	—	—	—	—

[a]The diet containing the indicated antibiotics was fed to the transgenic F0 larvae (heterozygous), and the antibiotic-free diet was used afterward.
[b]The F0 flies (1 day old) were crossed with their wild-type (WT) partners (4–7 days old) on an antibiotic-free diet for successive 10 days.
[c]The number of F1 female survivors on certain days relative to the total number of the newly emerged F1 females from three replicates. Different lower-case letters between the F1 survival rates from F0 females and males with the same antibiotic treatment indicate significant differences ($p < 0.05$, z-test).
[d]This female died on day 28.
[e]This female died on day 43.

For *D. melanogaster*, feeding Tet (10–1,000 μg/mL) to adults suppressed the Tet-off system-mediated embryonic lethality, while Dox was more efficient at identical concentrations (Horn and Wimmer, 2003). In *C. capitata*, feeding Dox instead of Tet at 100 μg/mL to adult flies completely suppressed embryonic lethality (Schetelig et al., 2009). Meanwhile, for the fsRIDL strain of the malaria mosquito *Anopheles stephensi*, feeding less than 10 μg/mL of Tet to the larvae was not sufficient to completely rescue the flightless phenotype of the females (Marinotti et al., 2013). For the *Aedes aegypti* OX513A strain, feeding 1 μg/mL Dox to the larvae fully rescued the pupal lethality and flightless phenotype, which was about 100 times more effective than Tet (Curtis et al., 2015). Here, we showed that feeding Dox, but not Tet, to *D. suzukii* F0 larvae led to suppression of female lethality in F1 (Figure 3). Together these results suggested that Dox is a more efficient suppressor molecule than Tet for drosophilid, tephritid, and mosquito species. Interestingly, for *Aedes aegypti* OX513A, adult male or female receiving 50 or 100 μg/mL chlortetracycline only produced very few adult progeny (Curtis et al., 2015), suggesting that either the antibiotics were not passed to the offspring or they were inherited but not able to suppress the engineered lethality. Since the lethality of OX513A strain is based on the accumulation of tTA which kills mosquitoes at the larval stage (Phuc et al., 2007; Curtis et al., 2015), it is possible that the chlortetracycline was inherited but degraded and therefore not able to bind and deactivate tTA binding at a later stage. On the other hand, in the early-lethal systems in *D. melanogaster* and *C. capitata* based on embryonic-specific promoters, parental antibiotics were suspected as a possible cause for the suppression of the embryonic lethality (Horn and Wimmer, 2003; Schetelig et al., 2009).

Feeding Tet or Dox to F0 mothers generated long-lived F1 female survivors in the FK strain V229_M4f1

In the previous study, several *Drosophila suzukii* female-killing lines produced few female adults when parental males were fed with antibiotics at larval stages, but survivors quickly died (Schetelig et al., 2021). For example, the female lethality in the strain V229_M41f1 reached 100% at day 1 after the emergence (1 day), whereas for strain V229_M37f2 and V229_M39m1, it reached 100% at day 3. However, for the strain V229_M4f1, the female lethality was only investigated on day 1 but not beyond that stage in the earlier study. Here, we counted the survival rates of the V229_M4f1 females that derived from antibiotic-treated (at the larval stage) mothers or fathers. When Tet-100 was added to the diet, 5% and 0.4% of females related to the treated fathers survived until days 2 and 4 after emergence, respectively, which were significantly lower than those from treated mothers ($p < 0.05$, z-test) (Table 1). All females derived from Tet-treated fathers died until day 6, while three females from Tet-treated mothers survived until day 6 and one female until day 28. When Dox-100 was applied, 3% of the females descending from treated fathers survived until day 2 and all died on day 4, while significantly higher survival rates at these days (21.4% and 5.8%) were found in the crosses using the treated mothers ($p < 0.05$, z-test). When Dox-250 was applied, no females from the treated fathers survived until day 2, whereas 18 females from the treated mother survived to this day and one female until day 43.

It appears that line V229_M4f1 kills females through the development, and a complete penetrance of the lethal phenotype can be achieved at a young adult stage when only feeding antibiotics to the father. On the other hand, feeding antibiotics to the mother may be responsible for the "escaper" of the lethal system that survived to the late adult stage. Treated mothers may load more antibiotic molecules into some eggs than others, so they become more resistant to the lethal system. Therefore, strategies like fsRIDL or TESS, which generated male-only populations, might be more advantageous than bi-sex lethal systems, which do not eliminate females in the release generation. Several release programs for mosquito control combined the bi-sex lethal RIDL strains with the sexing procedure that mechanically removes most females (Harris et al., 2011; Carvalho et al., 2014). However, for the insect species without an efficient sexing method, bi-sex lethal systems need to be carefully evaluated for the effect of maternal antibiotics on the penetrance of the killing effect.

TABLE 2 Effects of antibiotics on the male production of the *Drosophila suzukii* FK strain V229_M4f1.

Antibiotic[a] (concentration)	F0 flies[b]	No. of F1 males[c]		
		FL	Non-FL	Total
Tet +/− (100 μg/mL)	Female	96 ± 18 ab	106 ± 18 ab	202 ± 35 b
	Male	126 ± 23 a	136 ± 31 a	262 ± 51 ab
Dox +/− (100 μg/mL)	Female	79 ± 4 ab	101 ± 16 ab	180 ± 16 b
	Male	83 ± 10 ab	97 ± 33 ab	180 ± 43 b
Dox +/− (250 μg/mL)	Female	24 ± 1 c	44 ± 18 b	68 ± 19 d
	Male	63 ± 16 bc	81 ± 6 ab	144 ± 21 bc
WT (no antibiotics)	—	—	—	297 ± 13 a

[a]The diet containing the indicated antibiotics was fed to the transgenic F0 larvae (heterozygous), and the antibiotic-free diet was used afterward.
[b]The F0 flies (1 day old) were crossed with their wildtype (WT) partners (4–7 days old).
[c]The number of the newly emerged F1 males on certain days (after eclosion) was scored. Different lower-case letters in the same column indicate a significant difference ($p < 0.05$, one-way ANOVA).

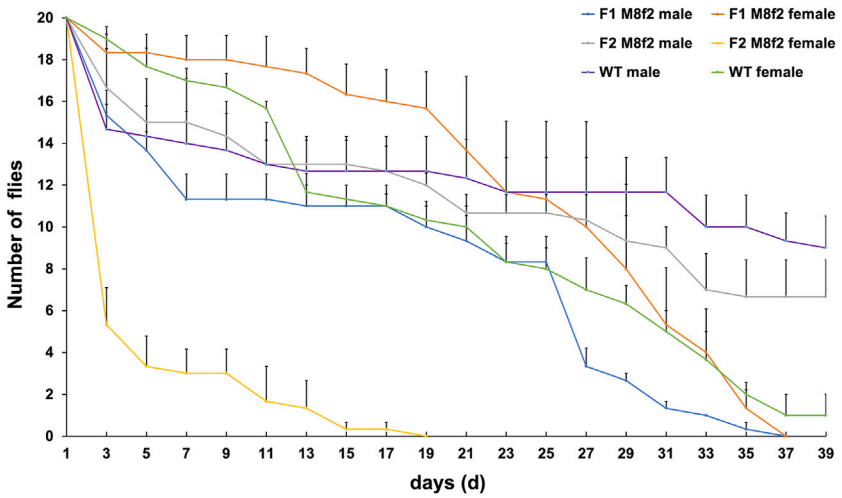

FIGURE 4
Transgenerational effect of antibiotics on fly longevity in the *D. suzukii* FK strain V229_M8f2. V229_M8f2 was previously generated by the germline transformation using the vector *V229_pBXLII_attP_PUbAmCyan_Dsnullo-tTA-SV40_TREhs43-Dshid^{Ala4}-CctraF-SV40* (Schetelig et al., 2021). Tet-100 diet was fed to V229_M8f2 F0 larvae but not to later stages. F0 or F1 females were crossed with the WT males, and newly emerged transgenic F1 and F2 flies were sexed and separated into fresh diet vials (20 flies per vial). These males or females were counted every 2 days before transferring to new vials until day 40 or all flies died. WT flies from the non-Tet diet were counted similarly. The mean and standard errors from three replicate experiments are shown.

Feeding Tet or Dox to F0 flies showed different effects on the F1 male production in the strain V229_M4f1

Similarly, we also counted the males derived from antibiotic-treated V229_M4f1 heterozygous mothers or fathers. Among all treated crosses, the Tet-100 fathers produced the highest number (262 ± 51) of male adult offspring, which was similar to that of WT ($p = 0.410$, one-way ANOVA). In contrast, the Dox-250 mothers produced the lowest number of males (68 ± 19), which was significantly lower than those from all other crosses ($p < 0.05$, one-way ANOVA; Table 2). In addition, all treated mothers and Dox-treated fathers produced fewer males compared to WT crosses ($p < 0.05$, One-way ANOVA), suggesting that the antibiotic residues from those treatments harmed the male survival throughout development. More specifically, the number of transgenic males descending from Dox-250-fed heterozygous mothers and fathers was significantly lower than that from Tet-100-fed mothers and fathers, respectively ($p < 0.05$, one-way ANOVA). Meanwhile, the number of WT male siblings in Dox-250 and Tet-100 were not significantly different ($p > 0.05$, one-way ANOVA). These observations indicated that the male survival in the transgenic files was more sensitive to increased doses of Dox than that in the WT flies, possibly due to fitness costs of the transgene. Male production is an important parameter to evaluate the suitability of insect strains for an SIT program. The results

suggested that feeding Dox to the *D. suzukii* FK strains at higher concentrations may reduce male production.

Feeding Tet to F0 mothers delayed the lethal effect for one generation in the FK strain V229_M8f2

It was recognized that the parental antibiotics might suppress the lethality correlated with the pro-apoptotic gene's activity or expression level (Horn and Wimmer, 2003; Schetelig et al., 2009; Yan and Scott, 2015; Yan et al., 2017). The latter is further subjected to the positional effects of the transgene (Schetelig et al., 2016; Concha et al., 2020; Yan et al., 2020c). It is possible that the low female lethality that we previously observed in some V229 lines was due to the low or moderate activity of the pro-apoptotic gene that was further suppressed by parental Tet (Schetelig et al., 2021). Therefore, we selected line V229_M8f2 that showed the lowest female lethality (32.7%) among seven V229 lines in the earlier study for a transgenerational longevity test. Similarly, Tet-100 was fed to F0 larvae, and F0 or F1 females were crossed with WT males on non-antibiotic food. We found that the survival rates of the F2 males on days 11, 21, and 31 were 65.0%, 53.3%, and 45.0%, respectively, which were not significantly different from those of WT males ($p > 0.05$, one-way ANOVA). Meanwhile, the survival rates of the F1 females on days 11, 21, and 31 were 88.3%, 68.3%, and 26.6%, respectively, which were not significantly different from those from WT females ($p > 0.05$, one-way ANOVA). However, most of the F2 females died on day 3, indicated by the 26.7% survival rate, which was significantly lower than those from the F1 females (91.7%) or WT females (95%) ($p < 0.05$, one-way ANOVA). This resembled the lethal adult stage in the F1 generation that we observed in most V229 lines (Schetelig et al., 2021). Additionally, all F2 females died on day 19, indicating that the full penetrance of the lethality can be achieved for this line after one additional generation after the last antibiotic treatment.

A previous study also reported that for *D. melanogaster* strains carrying a Tet-off-mediated fsRIDL system, feeding parents with 10 μg/mL Tet was as efficient as 100 μg/mL Tet in suppressing the female lethality in F1. However, the latter delayed the female lethality for one generation (Upadhyay et al., 2022). Homozygous strains were stably kept for recently developed transgenic sexing strains in *D. suzukii* using the pro-apoptotic gene $Dmhid^{Ala5}$ or *Dsrpr* as lethal effectors on diet with 20 μg/mL Tet (Li et al., 2021). Therefore, a low dose of Tet rather than a high dose may be preferred for *Drosophila* species in such laboratory experiments. This would provide a better assessment toward the induced lethality from different strains by minimizing the effects of the antibiotics.

Conclusion

For genetic pest control strategies such as RIDL or TESS, a proper Tet-feeding scheme that responsively and efficiently controls the lethal system is critical for applications in the mass-rearing and field release programs. Since those lethal systems could be leaky due to the insufficient or degraded Tet that does not entirely suppress the expression of the lethal gene (Thomas et al., 2000; Horn and Wimmer, 2003; Schetelig et al., 2009; Marinotti et al., 2013; Tan et al., 2013; Curtis et al., 2015; Yan and Scott, 2015; Schetelig et al., 2016; Concha et al., 2020), it is attractive to use higher doses of antibiotics for the maintenance of stock populations to counteract such potential leakiness of lethality. Here, we showed that while a high dose of antibiotics reduced the expression of Tet-off constructs to minimal in cell culture experiments, such treatments may have a profound effect on the performances of the *D. suzukii* transgenic strains employing the Tet-off system. We verified that feeding Dox but not Tet to F0 flies suppressed the F1 female lethality in the FK strain V229_M4f1. We also found that the parents treated with Dox produced significantly less transgenic male offspring than those from other antibiotic treatments. A genome-wide study showed that among several Tet derivatives, Dox elicited the most significant responses in the gene expression in *Saccharomyces cerevisiae* (Sanchez et al., 2020), and it also substantially alters cellular metabolism and impairs mitochondrial function (Luger et al., 2018). Therefore, although Dox is a more efficient suppressor molecule than Tet, it might undermine the lethal system at the designated stage and have a more severe impact on strain fitness.

The Tet-off constructs for *in vitro* analysis contain the same driver cassette (*Dsnullo-tTA*) and TRE-hs43 as in the V229 construct that was used to generate the FK strains V229_M4f1 and V229_M8f2. The only difference is the effector part. While the 2A peptide reporter cassettes serve as visual markers for the *in vitro* experiments, the pro-apoptotic effector gene $Dshid^{Ala4}$ in V229 allows us to evaluate the transgene expression by scoring the female lethal phenotype. Collectively, we showed that (more) female survivors could rise in our FK strains under certain antibiotic treatments. For the strain V229_M4f1 which showed moderate transgene activity, feeding Dox to F0 males or females suppressed the female lethality in F1, and feeding Tet or Dox to F0 females (but not males) generated long-lived F1 females. For the strain V229_M8f2 which showed weak transgene activity, feeding Tet to F0 females delayed the female lethality to F2. The suppression of F1 lethality observed in our FK strains was likely due to the effects of antibiotics that inherited in this generation, as previously suggested in several studies (Horn and Wimmer, 2003; Schetelig et al., 2009; Ogaugwu et al., 2013; Yan and Scott, 2015; Upadhyay et al., 2022). Another plausible reason for the suppressed lethality that is linked to the parental sex is the parental effects such as parental imprinting. Parental imprinting refers to the phenomena that the expression of an allele differs based on the sex of the parent that transmitted the allele (Lloyd, 2000; Lemos et al., 2014). In *D. melanogaster*, imprinting is typically associated with heterochromatin or regions with specific chromatin structures and often leads to epigenetic silencing (Abbott et al., 2013; Lemos et al., 2014; McEachern et al., 2014). It is possible that the transgene in the tested V229 strains was inserted into a genomic region with an imprinting-prone chromatin structure; therefore, the male parent transmitted an active form of the transgene, whereas the female transmitted an inactive silenced form which led to F1 survivors (Table 1). In addition, transgenerational imprinting was also observed in *D. melanogaster* (Oh et al., 2020), which might be associated with the one-generation delayed lethality in the strain V229_M8f2 (Figure 4). Further studies can be conducted to investigate the effects of the parental imprinting on the transgene-based lethal systems, such as verification of the genomic site of the transgene and its chromatin structure, comparison for the epigenetic silencing of the transgene from parental male and female, and transgenerational effects of such imprinting on the transgene activities.

The potential resistances to the Tet-off-based lethal system likely depend on the mechanism of lethality as well as transgene activity from each certain strain. For practical application, strains with strong engineered lethality (but otherwise fit) might be preferred over weak ones for a high or complete penetrance of lethality in the mass-rearing scenario. The strong or redundant lethality could help to counteract the pre-existing resistance in the wild populations (Knudsen et al., 2020). However, it may also speed up the generation of the second-site resistance mutations which caused the genetic breakdown of RIDL lethality in *D. melanogaster* (Zhao et al., 2020). Therefore, incorporating two or more lethal effectors/systems into the insect strains intended for use may help ensure the complete penetrance of lethality and reduce the development of resistance (Jaffri et al., 2020; Yan and Scott, 2020; Li et al., 2021; Upadhyay et al., 2022). For any genetic control strategies employing the Tet-off system, excessive use of Tet or its derivatives could reduce the strain fitness or lead to survivors, which may cause undesired damage or reduce the control efficiency. Therefore, the overall effects of antibiotics, including parental and transgenerational effects, on the engineered lethality and insect fitness need to be carefully evaluated in a species- and strain-specific manner for a safe and efficient practical application. Together with other genetic control programs such as SIT and incompatible insect technique (Nikolouli et al., 2018; Sassù et al., 2020), genetically engineered lethal strains could facilitate the sustainable management of this global pest *D. suzukii*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Author contributions

YY, BH, AS, SP, and TR performed the research. YY conceived the study and analyzed data. YY and MFS coordinated the project and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Handling Editor F.M. declared a past collaboration with the Author M.F.S.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2023.876492/full#supplementary-material

SUPPLEMENTARY TABLE S1
Antibiotics effects on live cells.

SUPPLEMENTARY FIGURE S2
ELISA analysis to measure antibiotics in *D. suzukii* adults and eggs.

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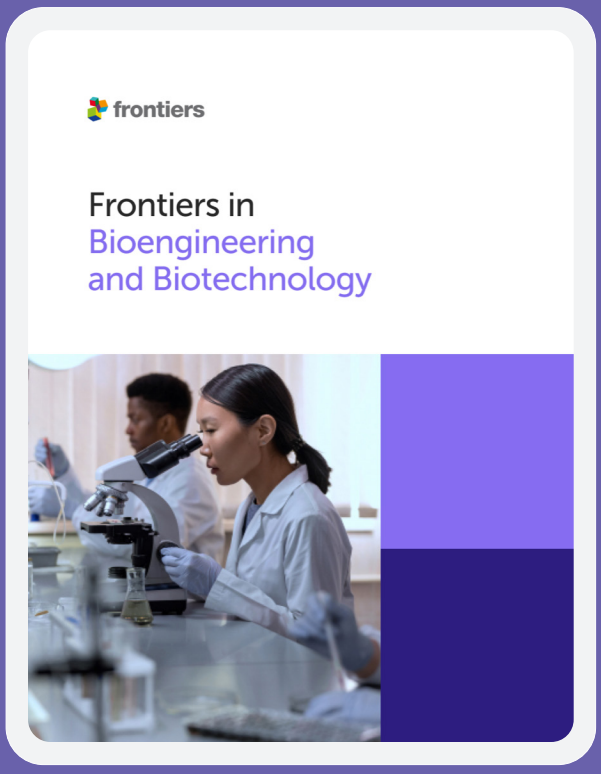

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