

SPECIAL ISSUE ARTICLE

Neoclassical development of genetic sexing strains for insect pest and disease vector control

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Abstract The sterile insect technique, which consists of the mass production and release of sterile insects to control populations of pests and disease vectors, has been effectively used for decades. An important component of sterile insect technique field applications is the availability of sex separation systems that reliably and economically eliminate females from mass-reared sterile insect populations destined for field release. Genetic sexing strains are important for the effectiveness and cost-efficiency of insect population control programs, including sterile insect technique. Classical approaches to generate genetic sexing strains, such as irradiation-induced chromosomal translocations, have yielded stable strains for species like the Mediterranean fruit fly, *Ceratitis capitata*. However, significant efforts are needed to establish genetic sexing strains using classical genetic methods, as large-scale random mutagenesis and screening are needed. We introduce here a neoclassical genetic approach, leveraging CRISPR-based gene-editing to target known genes to develop selectable genetic markers, followed by genetic rescue in a male-specific manner to speed up the development of genetic sexing strains and enhance their precision, stability, and adaptability. The integration of molecular tools, genetic markers like the *white pupae* and *temperature-sensitive lethal*, and strategies for maintaining genetic stability are discussed. We also review the challenges and opportunities in applying classical, transgenic, and neoclassical genetic approaches to improve genetic sexing strains for pest management.

Key words cardinal; CRISPR; gene editing; sterile insect technique; temperature-sensitive lethal; white pupae

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Introduction

Insect pests have a negative impact on several aspects of human life, affecting the environment, economy, and health (Vreysen *et al.*, 2006a). For crop production, yearly global losses are estimated to reach \$470 billion (Sharma *et al.*, 2017b). Furthermore, annual losses of \$4.75 billion are attributed to livestock pests (Kioy

et al., 2004; Van den Bossche et al., 2010). Besides economic losses, insect disease vectors can also affect human health, resulting in approximately 700 000 deaths annually (WHO, 2024). Insecticides have been used intensively to limit insect pest populations. While effective in the short term, excessive insecticide usage leads to long-lasting detrimental environmental consequences and the selection of insecticide resistance (Hawkins et al., 2019; Sharma et al., 2019; Hendrichs et al., 2021; Tudi et al., 2021). Considering these drawbacks, there has been significant investment in alternative control strategies that could be used in area-wide integrated pest management (AW-IPM) programs (Hendrichs et al., 2021).

Several insect control strategies have been developed in recent decades, including sterile hybrids, RNA interference (RNAi), behavioral manipulation and biological control. These methods employ the genetics of insect pests, natural mating systems or ecological niche to sterilize, modify, or eliminate them, thereby resulting in population suppression (Szendrei & Rodriguez-Saona, 2010; Cock et al., 2016; McFarlane et al., 2018; Vogel et al., 2019; Leftwich et al., 2020; Singh et al., 2022; Yan et al., 2023). However, these methods often lack scalability, specificity, or operational robustness necessary for AW-IPM programs. The most widely used method in AW-IPM is the sterile insect technique (SIT), first proposed by Knipling (1955). SIT is based on the mass production and release of irradiation-sterilized insects, ideally males, which should compete for mating with the males of the target wild population. Wild females mated with released, sterile males will not produce viable offspring, leading to population decline and collapse. SIT can be used for the suppression, containment, prevention of establishment, or the local eradication of an insect pest or disease vector species (Dyck et al., 2021).

SIT was first applied in the 1950s against the New World screwworm *Cochliomyia hominivorax* (Coquerel) in North and Central America, and later in Libya (Baumhover et al., 1955; Lindquist et al., 1992; Klassen et al., 2021). Since then, it has been applied against many different pest species, including its successful application for the eradication of the tsetse fly *Glossina austeni* (Newstead) from Unguja Island in Zanzibar, Tanzania (Vreysen et al., 2000), the melon fly *Zeugodacus cucurbitae* (Coquillett) from Japan (Kuba et al., 2020) and the Queensland fruit fly *Bactrocera tryoni* (Froggatt) from Western Australia (Sproule et al., 2001). These effective SIT applications involved releasing both sterilized males and females.

However, as has been demonstrated for the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), male-only releases can enhance the efficacy and

cost-effectiveness of SIT (McInnis et al., 1994; Hendrichs et al., 1995). This is due to several reasons: sterilized females of insect plant pest species can still cause damage to plant crops, for example when laying unfertilized eggs into host fruit, leading to cosmetic damage or fungal, bacterial and viral infections (Hendrichs et al., 1995), while in insect disease vectors, male-only releases are even more critical as blood-feeding females can potentially transmit pathogens (Papathanos et al., 2009; Gilles et al., 2014; Papathanos et al., 2018; Lutrat et al., 2019). In bisexual sterile releases, released males tend to mate with co-released females, instead of seeking wild counterparts, thus reducing the effectiveness of the SIT application (Vreysen et al., 2006b; Flores et al., 2014). Furthermore, in male-only releases, costs and logistics associated with post-production processes are reduced, as only half the volume of insects is handled for marking and irradiation. In addition, emergence, feeding to maturation, release, and monitoring activities are also significantly reduced (Epsky et al., 1999). However, the limited sexual dimorphism in most insect species prevents sex separation at the scale necessary for effective SIT implementation. To this end, genetic sexing strains (GSS) have been developed using classical genetics or molecular engineering. GSS are insect strains developed such that males and females differentially express a marker, which allows for easy sex separation. This article reviews the approaches for developing GSS, beginning with classical genetics and followed by transgenic methods, including their pros and cons. Recent advancements in CRISPR-based genome engineering and insect genomics allow us to propose a novel and potentially more broadly applicable method: the neoclassical genetic approach. This method may enable faster development of GSS with enhanced characteristics and quality for insect pest control applications by facilitating not only the direct transfer of selectable traits between species but also their subsequent linkage to maleness.

Genetic sexing strains—the classical genetics approach

Development of a GSS is based on two key components: a selectable phenotypic marker with a dominant wild-type allele (i.e., puparium or eye color) and the linkage of the dominant, rescue allele to the male-determining region (maleness) to ensure its sex-specific activity. Classical or molecular genetic methods have been utilized to develop GSS (Franz et al., 2021; Häcker et al., 2021). Using classical genetics, GSS can be constructed in three steps (Fig. 1). The first step includes the identifi-

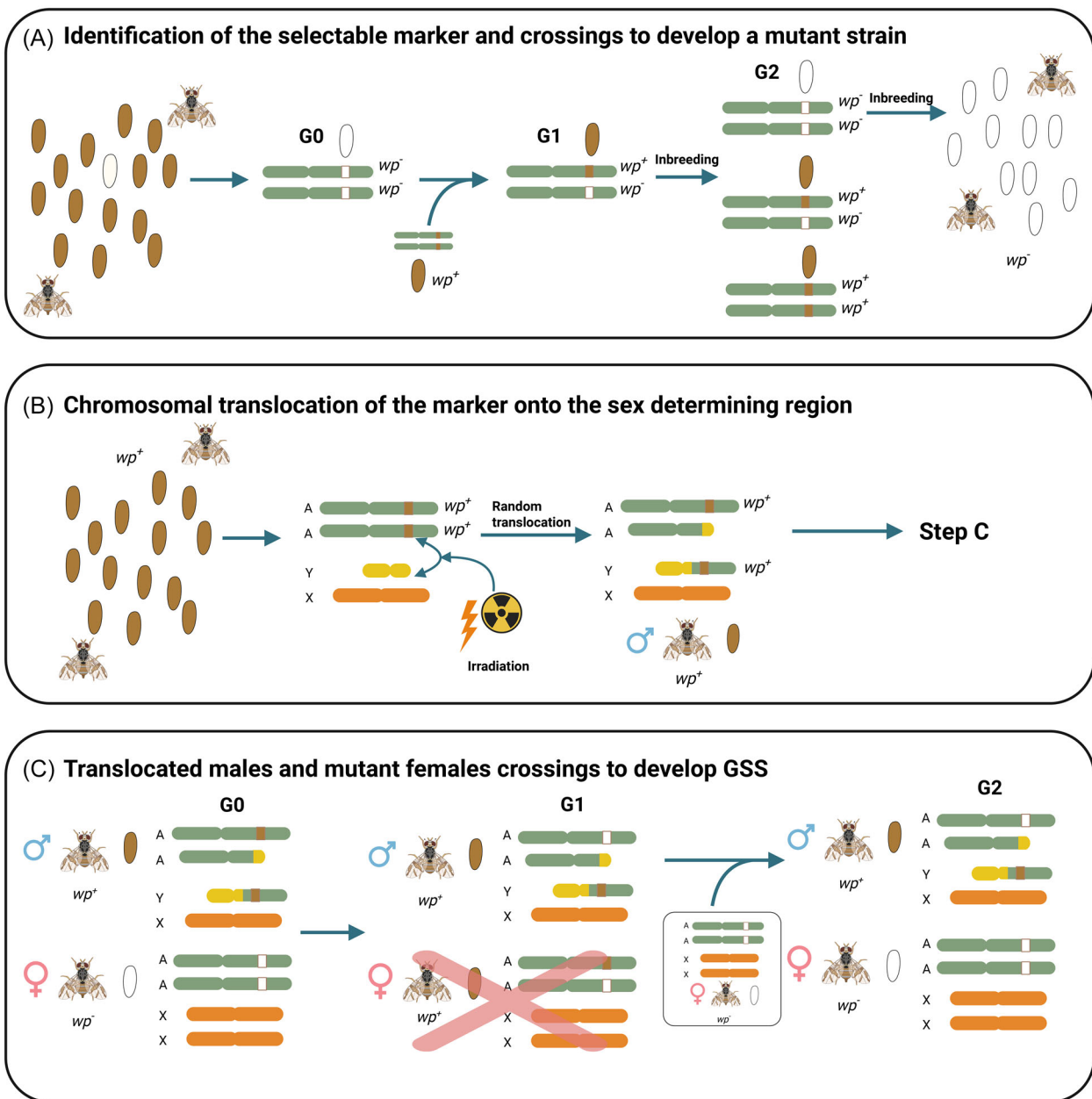


Fig. 1 Steps for developing classical genetic sexing strains (GSS). (A) Identify a recessive marker in a wild-type population (e.g., *white pupae*, wp), characterized by a visible or conditionally lethal trait (wp^+ : wild-type allele, brown pupae; wp^- : recessive mutant allele, white pupae in homozygotes). Such markers can also be induced via chemical mutagenesis (e.g., EMS) or irradiation. A mutant strain is then established, typically from a single G0 mutant crossed to wild type. Heterozygous G1 offspring are inbred, and G2 individuals with the mutant phenotype are selected and inbred to fix the trait. (B) Wild-type pupae (wp^+) are irradiated to induce reciprocal chromosomal translocations, aiming to link the marker locus to the Y chromosome. Due to the stochastic nature of this process, many individuals are treated. Emerged males are used for further crosses. (C) Irradiated males (from B) are crossed to mutant females (from A). G1 males are then crossed to mutant females. If the desired translocation occurred, G2 males will show the dominant phenotype (wp^+) and females the recessive phenotype (wp^-) (The translocated autosome and Y chromosome cannot segregate independently in classical GSS strains, as this would lead to genetic imbalance and non-viable sperm. See fig. 5 in Franz *et al.* (2021) for a complete outlook of the chromosomal segregations). If not, both phenotypes appear in both sexes (not shown). Created in BioRender. <https://BioRender.com/c90hdo1>. Schetelig, M., 2025.

cation of a selectable marker, which is usually associated with a visible trait. The marker can be found naturally in a population through screening, or it can be induced by chemical/irradiation-based treatments to wild-type strains. Following the identification of the marker of interest (e.g., pupae coloration), an appropriate scheme of crosses is performed to develop a homozygous mutant strain for the recessive allele (Fig. 1A). The second step involves linking the dominant, rescue allele of the marker to the male-determining region of the insect genome. The classical method to achieve this is by irradiation-induced chromosomal translocations (Fig. 1B). Among the irradiated insects, males in which the induced translocation resulted in the rescue allele being transferred onto the male-determining region (i.e., the Y chromosome for many insect species) are selected. These phenotypically wild-type male insects are then used to construct the GSS. The third step is the development of the GSS, where mutant females from the first step are crossed with wild-type males containing the translocated region from the second step (Fig. 1C). The resulting GSS strain will consist of males with the dominant wild-type phenotype and females exhibiting the recessive mutant trait. The most successful GSS ever constructed has been the VIENNA 8 GSS in the medfly, which are currently used in mass-rearing facilities and SIT programs worldwide (Cáceres *et al.*, 2004; Franz *et al.*, 2021).

The first generation of medfly GSS was based on loci determining the color of the puparium, using the *white pupae* (*wp*) mutation as a selectable marker (Rössler, 1979; Ward *et al.*, 2021). Later, in addition to the *wp* marker, a second marker was incorporated, the *temperature-sensitive lethal* (*tsl*) gene (Franz *et al.*, 1997; Robinson, 2002), with both markers being located on the right arm of chromosome 5 (Zacharopoulou *et al.*, 2017; Franz *et al.*, 2021). This second generation of GSS includes the VIENNA 7 and VIENNA 8 strains. In these GSS, females are homozygous for both mutations, emerge from white puparia, and do not develop when exposed to elevated temperatures as embryos (34–35 °C). On the other hand, the VIENNA 7 and VIENNA 8 GSS males are heterozygous for both mutations, emerge from brown pupae, and survive when exposed to high temperatures as embryos (Franz *et al.*, 2021).

Under small-scale rearing conditions in the laboratory, the VIENNA GSS seemed stable. However, when tested under mass-rearing conditions, signs of genetic instability were observed, resulting in recombinant individuals, which included males emerging from white pupae and/or being temperature sensitive and females emerging from wild-type (brown) pupae and/or being temperature resistant. Recombinant insects have a reproductive advan-

tage (females carry the wild-type alleles without fitness cost) and, therefore, can accumulate in the colony, ultimately leading to the loss of the sexing character of the GSS. The development of genetic and polytene chromosome maps allowed for a detailed genetic and cytogenetic analysis, which indicated type-1 male recombination as the main factor of genetic instability (Bedo, 1986; Zacharopoulou, 1987; Zacharopoulou *et al.*, 1991; Franz, 2002; Zacharopoulou *et al.*, 2017; Franz *et al.*, 2021). There are two forms of type-1 recombination: type-1a is observed between the translocation breakpoint and *wp*, while type-1b occurs between the two selectable markers, the *wp* and *tsl* genetic loci. A second, rare type of male recombination (type-2) has also been observed (Franz *et al.*, 2021). Moreover, VIENNA 8 males are semi-sterile, as only 50% of the produced sperm are genetically balanced after meiosis due to the translocation (Franz *et al.*, 2021). This is true for all classical GSS obtained *via* chromosomal translocations. GSS have now been developed for several other insect pest species, including *Anastrepha ludens* (Loew, 1873), *Anastrepha fraterculus* sp. 1 (Wiedemann), *Bactrocera dorsalis* (Hendel), and *Z. cucurbitae* using classical genetic approaches. In these GSS, genes that control the color of the puparium (*white pupae*, *black pupae*) were used as selectable markers (McCombs & Saul, 1995; McInnis *et al.*, 2004; Zepeda-Cisneros *et al.*, 2014; Meza *et al.*, 2020; Ramírez-Santos *et al.*, 2021).

Three different strategies have been employed to address the impact of recombination on the genetic stability of GSS. The first strategy included the selection of translocations where the breakpoint and the marker were as close as possible, thus minimizing the chances for recombination; sufficient productivity was also an additional criterion for selecting the best translocation (Keremans *et al.*, 1990; Keremans & Franz, 1995; Willhoeft & Franz, 1996; Franz *et al.*, 2021). Although it is very rare, reduction of type-2 recombination risk can be achieved by selecting translocations in which the translocation breakpoint on the Y-chromosome is located close to the centromere.

The second strategy involved the induction of inversions that cover the region between the translocation break point and the selectable marker(s). Chromosomal inversions are known as recombination suppressors. In a large screen for inversions, one such inversion was detected, namely D53 (Franz *et al.*, 2021). Although D53 covers only one of the two selectable markers, the *wp* gene, the recombination levels detected between *wp* and another marker called *Sergeant* (*Sr*²) that is located very close to *tsl* were low (Niyazi *et al.*, 2005; Franz *et al.*, 2021; Ward *et al.*, 2021). Combining the D53 inversion

with the translocation T(Y;5)101 resulted on the development of a new GSS strain called VIENNA 8^{+D53} (Augustinos *et al.*, 2017; Franz *et al.*, 2021).

The third strategy is using a Filter-Rearing System (FRS). An FRS requires a mother colony, which is constantly screened for the presence of recombinants. The mother colony is used as a founder for mass rearing colonies for SIT programs. To be effective, a FRS system requires the GSS to have an easily visible marker for the screening (e.g., the *white pupae* gene) (Fisher & Caceres, 2000). All these strategies have been successful in improving the stability of medfly GSS and have resulted in the successful deployment of the VIENNA 8 GSS, with or without the inversion D53, to operational AW-IPM programs that integrate SIT for the population control of medfly (Augustinos *et al.*, 2017).

Another issue is the genetic diversity of laboratory-reared GSS colonies. The mating behavior of laboratory-reared males can be negatively affected by inbreeding, and released males with reduced competitiveness can negatively impact SIT programs (Cayol, 1999). To minimize this, refreshing colonies with wild males to increase genetic diversity is recommended. This, however, proves to be particularly challenging as wild insects lack the irradiation-induced chromosomal translocation. To counteract this, a new GSS for *C. capitata* has been developed, which relies on a homozygous T(X;5) translocation of the *wp* and *tsl* markers (Cáceres *et al.*, 2023). Since the translocation is onto the X chromosome, colonies can be refreshed in only two generations. First, T(X;5) females are mated with wild-type males. From the resulting F1, males are backcrossed with the T(X;5) females, reestablishing the GSS.

In mosquito disease vectors, the first GSS constructed using classical genetic methods in the 1970s and 1980s, mainly for species of the *Anopheles* and *Culex* genera (Curtis *et al.*, 1976; Kaiser *et al.*, 1978; McDonald & Asman, 1982; Lines & Curtis, 1985; Malcolm & Mali, 1986; Shetty, 1987). All these strains relied on insecticide resistance genes linking resistance exclusively to males. In 2012, an *Anopheles arabiensis* (Patton) GSS was developed based on dieldrin resistance (Yamada *et al.*, 2012). However, the strain was not considered suitable for field use because of the genetic instability, low fertility, and concerns over dieldrin residues in adult males and subsequent environmental bioaccumulation (Yamada *et al.*, 2012; Yamada *et al.*, 2015).

More recently, GSS were developed for *Aedes aegypti* (Linnaeus) using classical genetics and mutations in eye color genes, *red-eye* and *white-eye*, as selectable markers (Koskinioti *et al.*, 2021). As both *red-eye* and *white-eye* genes are located on chromosome 1 (Chen *et al.*, 2022),

which contains the M locus that determines the male sex in this species, induction of translocations was not needed to construct these GSS. The red-eye GSS was proven to be genetically more stable and of higher quality than the white-eye GSS (Koskinioti *et al.*, 2021; Misbah-ul-Haq *et al.*, 2022b). Using radiation, a chromosomal inversion (Inv35) was induced, and incorporated in the red-eye GSS, reducing recombination rates between the marker and the M locus (Augustinos *et al.*, 2020). The red-eye GSS/Inv35 strain remained stable for over 15 generations without filtering out recombinants, with the recombination rate remaining below 1%. The sexing properties and genetic stability of the red-eye GSS (with and without Inv35) were confirmed in diverse genomic backgrounds (Augustinos *et al.*, 2022; Misbah-ul-Haq *et al.*, 2022a).

Taken together, productivity, genetic stability, and sexing properties are key factors that should be considered for the development and evaluation of any GSS to be used for the population control of insect pests and disease vectors. The procedure for developing GSS using classical genetic approaches is both labor- and time-intensive. Screening of many insects, possibly tens or hundreds of thousands (or even millions if a mutation is rare), is required to detect the desired phenotypic mutation. The chemical or irradiation treatments, for both isolating a suitable mutation and inducing the translocation, are stochastic and yield unpredictable results. For example, several groups spent more than two decades on research and evaluation to develop the medfly VIENNA GSS (Augustinos *et al.*, 2017). The availability of mutant strains, molecular markers, genetic linkage maps, polytene chromosome maps and other (cyto)genetic and molecular tools is of great importance for the development of classical GSS (Mavragani-Tsipidou *et al.*, 2014; Zacharopoulou *et al.*, 2017).

Genetic sexing strains—the transgenic approach

During the last three decades, advances in molecular biology and genetics have offered new approaches for developing GSS. To clarity, we refer to transgenic approaches here as those involving the introduction of at least one non-host derived DNA sequence into the target insect genome (Häcker *et al.*, 2021). As it is important to control the trait used for sexing during insect mass rearing, transgenic approaches considered several binary conditional systems, such as the GAL4-UAS system (Brand & Perrimon, 1993), the Q system (Potter *et al.*, 2010), and the “Tet-On/Tet-Off” systems (Gossen & Bujard, 1992;

Gossen *et al.*, 1995). Considering that the GAL4-UAS system requires keeping two strains in the rearing facilities, focus has been applied to the Tet-On/Tet-Off systems. The Tet gene expression system functions when a recombinant tetracycline-responsive transcription factor (called tTA for Tet-Off or rtTA for Tet-On) binds to the *TetO* promoter, driving the expression of the target gene. In insects, the first Tet-Off system was developed as a proof-of-principle in *Drosophila melanogaster* (Meigen) (Bello *et al.*, 1998). Later, Heinrich and Scott (Heinrich & Scott, 2000) established a conditional female-specific lethality system in *D. melanogaster*, causing death in late pupal or early adult stages. This was accomplished by expressing the pro-apoptotic gene *head involution defective* (*hid*) in the adult female fat body.

In 2000, Thomas and colleagues introduced the “Release of Insects carrying a Dominant Lethal” (RIDL) approach (Thomas *et al.*, 2000) using a Tet-suppressible RIDL system in *D. melanogaster*. In this system, rather than using a specific promoter to drive tTA, which subsequently activates a lethal gene, an autoloop was created in which tTA activates its own gene expression. This leads to the accumulation of toxic levels of tTA in the late larval or pupal stages in the absence of Tet. This system was designed to be sex specific, creating the so called female specific RIDL (fsRIDL) in medfly and mosquitoes (Thomas *et al.*, 2000; Fu *et al.*, 2007; Phuc *et al.*, 2007; Fu *et al.*, 2010; Harris *et al.*, 2011; Wise de Valdez *et al.*, 2011; Harris *et al.*, 2012; Labbé *et al.*, 2012). The fsRIDL is not a GSS for SIT applications but rather a standalone system that involves the release of fertile males. In this system in medfly, tTA acts as a lethal effector made female-specific by integrating a sex-specifically spliced gene intron of a *transformer* (*tra*) gene. In mosquitoes a female-specific actin promoter and a female-specific intron in the same actin gene were used. This results in female-specific lethality when tetracycline is absent from the diet. Tet-Off sexing strains, presenting female lethality during early embryonic stages, have been reported for *D. melanogaster* (Heinrich & Scott, 2000; Thomas *et al.*, 2000; Alphey, 2002), *C. capitata* (Gong *et al.*, 2005; Fu *et al.*, 2007; Schetelig *et al.*, 2009; Ogaugwu *et al.*, 2013), *Ae. aegypti* (Spinner *et al.*, 2022), *Anastrepha suspensa* (Loew) (Schetelig & Handler, 2012), *A. ludens* (Schetelig *et al.*, 2016), *Lucilia cuprina* (Wiedemann) (Yan & Scott, 2015; Yan & Scott, 2020), *C. hominivorax* (Concha *et al.*, 2016; Concha *et al.*, 2020), *Bactrocera oleae* (Rossi) (Ant *et al.*, 2012), and *Drosophila suzukii* (Mastsumura) (Schetelig *et al.*, 2021). Similarly, a drug based transgenic system relying on sex-specific alternative splicing was developed in *D. melanogaster* (Kandul *et al.*, 2020). It relies on a sex sorter cassette comprising two genes, *PuroR*

and *NeoR*, conferring resistance to the commonly used pesticides puromycin and geneticin. A male-specific intron from the sex determining gene *dsx* was inserted in the coding region of *PuroR*, allowing for correct splicing of the puromycin resistant gene only in males. At the same time a female-specific intron from the sex determining gene *tra* was inserted in the coding region of *NeoR*, conferring geneticin resistance only in females after correct splicing. Therefore, either only female or only male flies can be obtained by simply providing the correct antibiotic in the fly diet.

Transgenic GSS have also been constructed by inserting fluorescent markers close to the male determining region in fruit flies and mosquitoes, which would, in principle, allow the sorting of fluorescent individuals at early developmental stages using a fluorescence-based sorter thus releasing sterile males-only (Catteruccia *et al.*, 2005; Meza *et al.*, 2014; Bernardini *et al.*, 2018; Buchman & Akbari, 2019; Lutrat *et al.*, 2022; Ntoyi *et al.*, 2022; Davydova *et al.*, 2023; Lutrat *et al.*, 2023).

RNAi-based methods have been developed in mosquitoes, targeting sex determining genes such as *doublesex* to achieve sex sorting (Whyard *et al.*, 2015) or genes involved in neuromuscular development such as *Rbfox1*, acting as insecticides (Mysore *et al.*, 2021). Interestingly, RNAi-based insect control methods can or cannot be classified as transgenic, depending on how the RNA molecules are delivered to the target organism. While promising, RNAi-based approaches have been reported to have mixed efficiencies (Prates *et al.*, 2024), as the approach is not 100% efficient and is not easily scalable in mass rearing. For disease-carrying insects, such as mosquitoes, this incomplete efficiency makes the approach inherently unsuitable, as it might not meet the necessary requirements for biosafety standards.

Although transgenic methods can be applied to diverse insect species, and their effectiveness under laboratory conditions seems promising, their implementation in AW-IPM control strategies with an SIT component has been challenging. This is because of the introduction of recombined DNA from one organism to another, the variable levels of containment of the resulting transgenic organisms, and the discourse around their cultural and ethical dimensions, which have resulted in the development of a strict regulatory framework (Oye *et al.*, 2014; Häcker *et al.*, 2021). In addition, the biological quality and genetic stability of the transgenic strains produced so far have not been thoroughly tested under real mass-rearing conditions, while in some cases, their quality, as tested under laboratory or small-scale conditions, was unsuitable for field applications (Häcker *et al.*, 2021).

Gene editing and neoclassical GSS

The CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated protein) system has greatly advanced the field of genome editing, and is now widely employed in scientific research (Doudna & Charpentier, 2014; Nidhi *et al.*, 2021). This molecular tool was first discovered as part of the immune system of prokaryotic organisms (Mojica & Rodriguez-Valera, 2016). Upon infection from a pathogen, the prokaryotes incorporate a short sequence of the foreign DNA into their genome. When the same pathogen reinfects the prokaryotic organism, one family of Cas proteins transcribes and processes the sequences into short CRISPR RNAs (crRNAs), which then guide the Cas proteins to degrade the foreign DNA by endonucleolytic cleavage. The crRNA determines the site specificity of the Cas endonuclease. This characteristic gives great plasticity to the procedure by simply adjusting the sequence of the crRNAs. The procedure is cost-effective and gives the ability to induce DNA breaks to the DNA target sequence with extreme precision.

CRISPR-cleaved DNA can be repaired mainly by two DNA repair pathways: Non-homologous end joining (NHEJ) and homology-directed repair (HDR). In NHEJ, DNA is repaired by joining the cleaved ends. While simple, NHEJ can lead to repair errors resulting in DNA indels (Hsu *et al.*, 2014; Lino *et al.*, 2018). Because of this, CRISPR-induced NHEJ is exploited in knockout experiments to study gene expression and function or to generate organisms with a loss of function of a target gene. HDR instead relies on homologous donor templates delivered as single or double-stranded DNA (Hsu *et al.*, 2014; Lino *et al.*, 2018). This pathway can be exploited in genome editing to insert exogenous DNA into the target sequence. In insects, CRISPR/Cas-based genome editing was first tested in *D. melanogaster* (Ren *et al.*, 2013; Bassett & Liu, 2014; Yu *et al.*, 2014; Wang & Doudna, 2023) and has now been applied to several other insect species, including agricultural pests (Bi *et al.*, 2016; Koutroumpa *et al.*, 2016; Kalajdzic & Schetelig, 2017; Li & Handler, 2017; Meccariello *et al.*, 2017; Aumann *et al.*, 2018; Li & Handler, 2019; Sim *et al.*, 2019; Zhao *et al.*, 2019; Choo *et al.*, 2020; Ward *et al.*, 2021; Yan *et al.*, 2023; Asad *et al.*, 2025; Paulo *et al.*, 2025), disease vectors (Hall *et al.*, 2015; Kistler *et al.*, 2015; Hammond *et al.*, 2016; Chen *et al.*, 2022), economically relevant species (Ma *et al.*, 2014) and other model insect species (Markert *et al.*, 2016).

Considering the principles of the classical genetics approach and the rapid advances of molecular engineering methods, particularly involving CRISPR/Cas systems,

a new generic method for the development of GSS is proposed, namely the “Neoclassical Genetic Approach,” which is also the focus of the Joint FAO/IAEA Coordinated Research Project on “Generic approach for the development of genetic sexing strains for sterile insect technique applications” (FAO/IAEA, 1st RCM report, 2019; FAO/IAEA, 2nd RCM report, 2021; FAO/IAEA, 3rd RCM report, 2023) (<https://www.iaea.org/projects/crp/d44003>). The “Neoclassical Genetic Approach” aims to establish a generic work pipeline for quickly and reliably developing GSS, which can be divided into three steps (Fig. 2).

The initial step of the neoclassical genetic approach to develop a GSS for a SIT target species is the identification of the gene responsible for a certain phenotype and the characterization of the causal mutation, which will be used as a trait (selectable marker) for sex sorting in target species. Ideally, the gene should be encoding a visible or a conditional lethal trait. In case the genetic basis of the selectable marker is unknown, it can be revealed by an integrated approach combining molecular, (cyto)genetic, genomic, transcriptomic, and bioinformatic analysis. Once the genetic basis of the marker is identified, orthologs can be identified in other species (Fig. 2A). CRISPR makes it possible to develop selectable traits in different target species if the causal mutations are known. The next step is to induce the same or similar mutation(s) to the orthologs of this gene in the target species by genome editing, confirming the desired phenotype (Fig. 2B). Genome editing could then be used to insert (knock-in) the rescue (wild-type) allele into a well-defined sex-specific chromosomal region, either male- or female-specific, depending on the karyotype of the insect species. In an XY male system, the rescue allele would be inserted on the Y chromosome, enabling crosses with mutant females to establish the GSS (Fig. 2C1). Following this procedure, a new GSS strain could potentially be developed in any insect of interest if: (a) orthologs are present in the target species and maintain the same function; (b) unique sex-specific and transcriptionally active regions can be identified and (c) functional rescue alleles (or mini-genes) can be inserted by genome editing approaches. If (b) or (c) is proven challenging, radiation-induced translocations could be used to achieve the linkage (Fig. 2C2). The main advantages of the neoclassical approach compared to classical GSS are summarized in Table 1.

CRISPR/Cas-based gene editing plays a key role at all stages: (a) inducing mutations in candidate genes to uncover the genetic basis of phenotypes suitable for sex separation; (b) replicating or inducing similar mutations in the orthologs of selectable markers in other species via NHEJ; and (c) inserting a rescue allele of a selectable

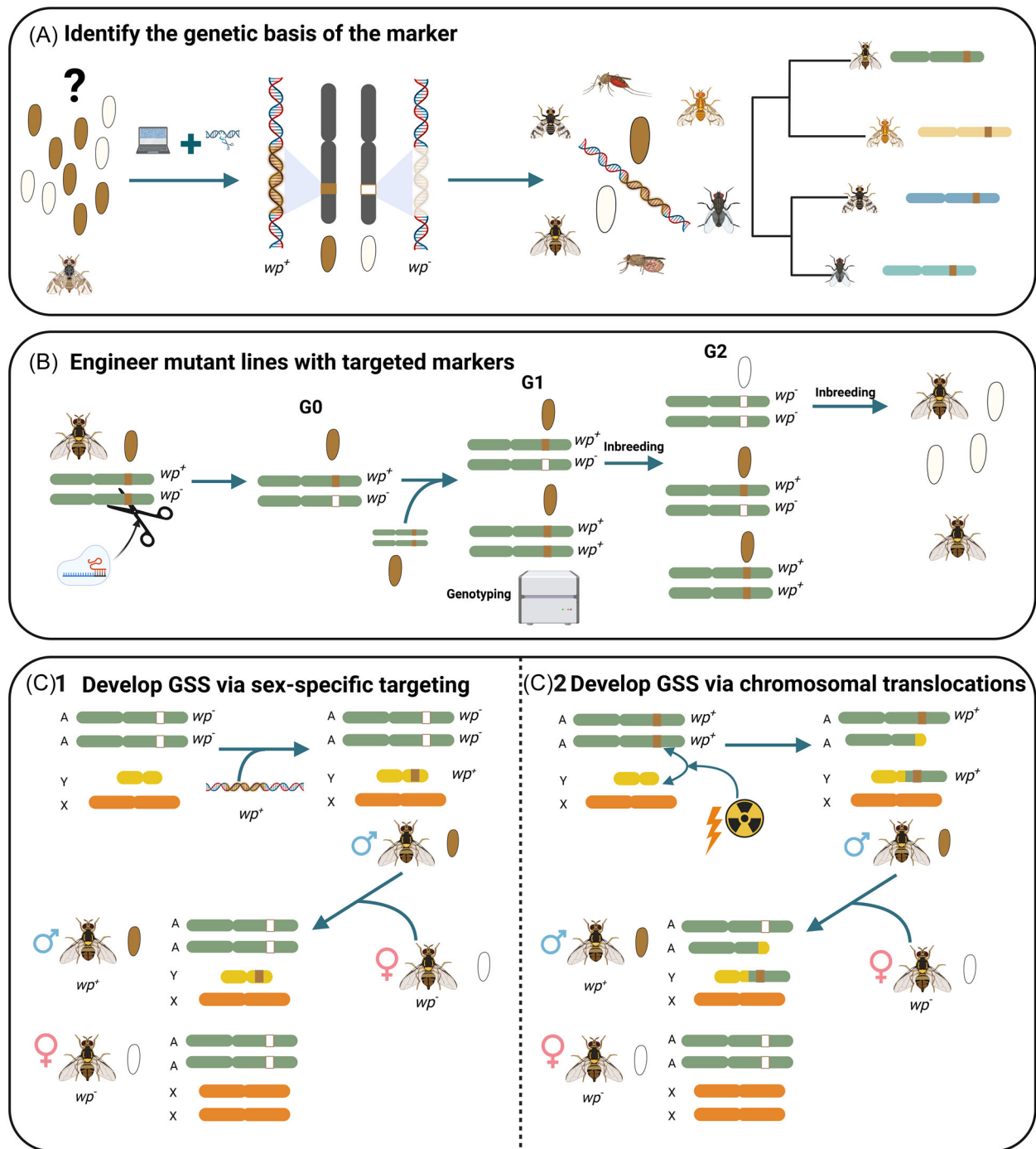


Fig. 2 Steps for developing neoclassical genetic sexing strains (GSS) using a generic approach. (A) Identify the genetic basis of a visible or conditionally lethal marker, such as *white pupae* (wp), through molecular, genomic, transcriptomic, and/or bioinformatic analyses, including functional knockouts. The wild-type allele (wp^+) produces brown pupae, while the recessive mutant allele (wp^-) produces white pupae in homozygotes. In species lacking such markers, orthologs can be identified through comparative genomics. (B) Introduce the recessive phenotype via CRISPR/Cas-mediated knockouts. G0 individuals are crossed to wild type to generate G1, which are genotyped to distinguish wp^+/wp^+ from wp^+/wp^- individuals. Heterozygotes are inbred, and homozygous wp^-/wp^- G2 progeny are selected based on the white pupae phenotype and inbred to establish the mutant strain. (C) Establish a GSS by either of two strategies. In C1, a wp^+ rescue allele is inserted into the male-determining region (e.g., Y chromosome) of the wp^-/wp^- line,

Table 1 Common issues of classical GSS that can be addressed with the neoclassical GSS approach.

| Classical GSS | Neoclassical GSS |
|--|--|
| Recombination between the marker and the sex-determining region may occur, requiring constant screening or the use of stabilizing strategies such as filter rearing systems or chromosomal inversions. | Recombination is unlikely, as the rescue allele is tightly linked to the sex-determining region. |
| Semi-sterility is common due to chromosomal translocations; ~50% of gametes are genetically unbalanced and non-viable. | No semi-sterility, as genome integrity is preserved without translocations. |
| Development is slow and stochastic, depending on the identification of a suitable marker and successful induction of a translocation via irradiation. | Development is efficient and targeted; CRISPR/Cas enables precise generation of both the marker mutant and GSS without relying on random mutagenesis. |
| Colony maintenance is labor- and resource-intensive due to the complex and unstable genetic background. | Colony management is simplified due to the stable and minimal genetic modifications; GSS can be rapidly reconstructed in different genomic backgrounds if necessary. |

marker using the HDR-based knock-in. When linked to the male-determining region, the rescue allele enables sex-specific expression, facilitating GSS development. CRISPR/Cas-based editing has been successfully applied in the first two steps, identifying the genetic basis of *white pupae*, *black pupae*, and *red-eye* phenotypes in tephritid and mosquito species, and inducing similar mutations in the same or other pest species (Ward *et al.*, 2021; Chen *et al.*, 2022; Paulo *et al.*, 2025). While several candidate genes for the temperature-sensitive lethal phenotype have been identified and studied, including *deep orange* and *shibire* (Choo *et al.*, 2020; Sollazzo *et al.*, 2023; Sollazzo *et al.*, 2024), the *lysine-tRNA ligase* (*Lysyl-tRNA synthetase LysRS*) gene has recently been identified as the most promising candidate for generating tsl strains (Aumann *et al.*, 2025). In *C. capitata*, a single H > Y mutation in exon 5 of this gene induced the tsl phenotype. Moreover, temperature sensitivity was successfully rescued with a *LysRS* minigene, demonstrating that this gene is indeed responsible for the phenotype. Further studies will prove if the same point mutation can be induced in other species to obtain temperature-sensitive lethal strains. Interestingly, complete gene knockout and single point mutations can produce different phenotypes, as the second one leads to structural rearrangement of the protein while the first to early disruption of protein assembly. For example, *LysRS* with a single point

mutation displays a tsl phenotype, while no knockout individuals were observed, suggesting that knockout of *LysRS* is lethal. Still, gene knockout studies are more easily achieved, as NHEJ events are easier to generate than HDR, even for single-pair substitutions. There are ongoing efforts to link the wild-type allele of selectable markers to the male-determining region of target species using CRISPR/Cas9-based approaches (FAO/IAEA, 2nd RCM report, 2021; FAO/IAEA, 3rd RCM report, 2023).

However, identifying a suitable region for inserting the rescue allele may be challenging. Several male-determining genes have been identified in insect pests and disease vectors, including *Nix* in *Ae. aegypti*, *Aedes albopictus* (Skuse), and other *Culicinae* species (Hall *et al.*, 2015; Liu *et al.*, 2020; Biedler *et al.*, 2024), *Yob* in *Anopheles gambiae* (Giles) (Krzyszewska *et al.*, 2016), *Guy1* in *Anopheles stephensi* (Linston) (Criscione *et al.*, 2013; Criscione *et al.*, 2016), *Mdm* in *Musca domestica* (Linnaeus) (Sharma *et al.*, 2017a) and the *Maleness-on-the-Y* (*MoY*) gene in *C. capitata* (Meccariello *et al.*, 2019). However, assembling chromosomes or chromosomal regions containing these genes remains challenging due to their highly heterochromatic and repetitive nature (Tomaszkiewicz *et al.*, 2017). Continuous progress in next-generation sequencing technologies and bioinformatics may overcome the hurdles toward the identification of proper anchor region(s), which should: (a) be

producing males with a $wp^+/wp^-/wp^-$ genotype and dominant phenotype. In C2, the wp^+ allele is translocated onto the male-specific chromosome via irradiation. Males with the desired translocation are crossed with wp^-/wp^- females to establish the GSS. Created in BioRender. <https://BioRender.com/w31a565>. Schetelig, M., 2025.

unique in the genome of the insects, (b) be transcriptionally active, as this is crucial for the correct temporal and spatial expression of the wild-type allele of the selectable marker, and (c) not disrupt essential genes. The insertion of the rescue allele may also be challenging. While targeted HDR has been successfully implemented in different insects, including *C. capitata*, *D. suzukii*, *B. tryoni*, *B. dorsalis*, *Ae. Aegypti*, and *An. gambiae* (Hammond et al., 2016; Li & Handler, 2017; Aumann et al., 2018; Ahmed et al., 2019; Choo et al., 2020; Lutrat et al., 2023; Teng et al., 2024), insertion in highly repetitive and heterochromatic chromosomal regions has proven to be challenging but doable, as *D. melanogaster* expressing fluorescent markers and Cas9 from Y specific knock-ins have been developed (Buchman & Akbari, 2019; Gamez et al., 2021). Mini-genes that can fully rescue the wild-type phenotype may need to be developed. Mini genes are highly compact gene versions, often reduced to coding exons, which facilitate successful HDR or improved transgenesis due to a smaller cargo (Roseman et al., 1993; Silicheva et al., 2010; Prates et al., 2025). However, this may not be sufficient to address the challenge of integrating and expressing the rescue gene into the Y-chromosome.

Selectable markers for the neoclassical approach—genes and causal mutations

Different selectable markers could be used for the construction of GSS. Regardless of the type of marker (visible or not), it is important to be monogenic, stable, with full penetrance and expressivity. The rescued marker should display a distinctly dominant phenotype. Ideally, the marker should be expressed as early as possible during the development of the insect. For example, the *tsl* marker of the *C. capitata* VIENNA 8 GSS is expressed throughout development, starting at early embryonic stages. It allows efficient sex sorting, eliminating the females by incubating embryos at elevated temperatures. In this way, the overall costs of an SIT program are minimized, and the accuracy and practicality of the procedure is optimized. Interestingly, temperature-sensitive lethal phenotypes have been reported in many species and are often found to result from a single amino acid substitution in a polypeptide, which alters protein activity, function, or stability at varying temperatures. However, temperature sensitivity may also arise from different types of mutations, including chromosomal ones, and it could potentially stem from synonymous mutations as well (Aumann et al., 2020; Choo et al., 2020; Shen et al., 2022; Sollazzo et al., 2024). While the *C. capitata* *tsl* marker

has been successfully employed to develop classical GSS, its genetic basis has remained largely unknown until recently. In *D. melanogaster* the *tsl* phenotype is correlated with independent mutations in different genes (Nguyen et al., 2021). Genomic, bioinformatic, and cytogenetic analyses in *C. capitata*, followed by CRISPR/Cas9 gene editing, identified the *C. capitata* *deep orange* (*Ccdor*) as a candidate target for the development of *tsl*-based GSS (Sollazzo et al., 2023; Sollazzo et al., 2024). Although *dor* temperature-sensitive lethal mutant lines do not exhibit the same *tsl* phenotype as in *C. capitata*, the gene's high conservation suggests its potential as a marker for developing neoclassical GSS in other species. Another candidate gene for GSS development is *shibire*. A single G to A point mutation of this gene, which is involved in synaptic vesicle formation, is enough to determine the *tsl* phenotype in *D. melanogaster*. The same point mutation was induced in the *B. tryoni* homologous *shibire* gene, resulting in a similar phenotype (Choo et al., 2020). For the widely successful *C. capitata* GSS strain Vienna 8, the causal mutation of the *tsl* phenotype has recently been identified as a single point mutation in the *LysRS* gene (Aumann et al., 2025) (Table 2 summarizes the markers that can be used for the development of neoclassical GSS).

The *tsl* marker appears to be ideal for sex sorting. However, it has limitations. Since it is not a visible marker, it cannot be used alone for mass-rearing and large-scale SIT applications because its stability cannot be easily monitored. Therefore, it should be linked with a second, ideally visible, selectable marker. In the case of the medfly VIENNA GSS, the *wp* gene plays this role, changing the wild-type brown color of the puparium to white (Rössler, 1979). *White pupae* mutations have also been discovered and used as selectable markers for the construction of GSS in two other tephritid species, *B. dorsalis* (McCombs & Saul, 1995) and *Zeugodacus cucurbitae* (McInnis et al., 2004). Using an integrated approach combining molecular, (cyto)genetic, genomic, transcriptomic and bioinformatic analysis, it was found that the white pupae phenotype of three tephritid species, *C. capitata*, *B. dorsalis*, and *Z. cucurbitae*, is caused by parallel mutations of a gene member of the Major Facilitator Superfamily (MFS) of membrane transporter proteins (Ward et al., 2021). This transporter is most likely involved in the mechanism responsible for transferring catecholamines from the hemolymph to the pupal cuticle (Wappner et al., 1995). The causal mutation in the *Ccwp* gene involves an insertion of a transposon-like element approximately 8150 bp long, whereas in the other two species, the mutation is a deletion: 37 bp in the *Bdwp* gene and 13 bp in the *Zcwp* gene (Ward et al., 2021).

Table 2 Markers that can be used for the development of neoclassical GSS.

| Phenotype | Validated marker (via gene editing) | References |
|--|--|--|
| Lethality or paralysis under high-temperature exposure | <i>shibire</i> (<i>D. melanogaster</i> , <i>B. tryoni</i>); <i>deep orange</i> , <i>LysRS</i> (<i>C. capitata</i>) | Choo <i>et al.</i> , 2020; Sollazzo <i>et al.</i> , 2023; Sollazzo <i>et al.</i> , 2024; Aumann <i>et al.</i> , 2025 |
| White puparium instead of brown | <i>white pupae</i> (<i>C. capitata</i> , <i>B. dorsalis</i> , <i>B. correcta</i> , <i>B. oleae</i> , <i>Z. cucurbitae</i> , <i>Z. tau</i>) | Ward <i>et al.</i> , 2021; Ioannidou <i>et al.</i> , 2025 |
| Black body coloration in immature or adult stages | <i>ebony</i> (<i>A. ludens</i> , <i>A. fraterculus</i> , <i>C. capitata</i> , <i>B. tryoni</i> , <i>B. dorsalis</i> , <i>Z. cucurbitae</i> , <i>Ae. aegypti</i> , <i>Culex quinquefasciatus</i> , <i>Plutella xylostella</i> , <i>Spodoptera litura</i>) | Zepeda-Cisneros <i>et al.</i> , 2014; Bi <i>et al.</i> , 2019; Meza <i>et al.</i> , 2020; Feng <i>et al.</i> , 2021; Xu <i>et al.</i> , 2021; Nikolouli <i>et al.</i> , 2025; Paulo <i>et al.</i> , 2025 |
| Red-eye color instead of black (in mosquitoes) | <i>cardinal</i> (<i>Ae. aegypti</i> , <i>An. gambiae</i>) | Carballar-Lejarazú <i>et al.</i> , 2020; Chen <i>et al.</i> , 2022 |

The *ebony* (*e*) gene, which was recently found responsible for the black pupae phenotype, could also be a useful selectable marker. Mutations in this gene change the brown color of the puparium to black in *Anastrepha*, *Bactrocera*, *Ceratitis*, and *Zeugodacus* species and have been used as markers for the construction of GSS in *A. ludens* and *A. fraterculus* *sp.1* (Zepeda-Cisneros *et al.*, 2014; Meza *et al.*, 2020; Paulo *et al.*, 2025). However, *ebony* mutations have been shown to have fitness costs in several species, including *B. tryoni* and *Ae. aegypti* (Nikolouli *et al.*, 2025; Paulo *et al.*, 2025). Generation of knockouts has also been reported for *Culex quinquefasciatus* (Say), *Plutella xylostella* (Linnaeus), and *Spodoptera litura* (Fabricius) (Bi *et al.*, 2019; Feng *et al.*, 2021; Xu *et al.*, 2021).

Another promising marker is the Sergeant (*Sr²*) gene. A mutation in this gene has been reported in the medfly, resulting in three white stripes on the abdomen of adult insects instead of the usual two (Niyazi *et al.*, 2005). This gene is located on the right arm of chromosome 5, closely linked to the *tsl* gene. The *Sr²* mutation is homozygous lethal in medflies. However, it could serve as a useful marker for monitoring the released sterile males in SIT applications and for discriminating them from the males of the target local population (Niyazi *et al.*, 2005). Until today, monitoring the released insects is based on the detection of powdered fluorescent dye used to coat pupae before adult emergence (Verhulst *et al.*, 2013; Clymans *et al.*, 2020). However, this is not ideal for several reasons: (a) if inhaled, these dyes can be harmful to workers in mass-rearing facilities; (b) the dye may be lost, potentially impacting post-release monitoring programs; and (c) in some species, the dye not only depletes over time, but it may also alter behavior, complicating efforts to estimate the competitiveness of the released males (Johnson *et al.*, 2017; Aviles *et al.*, 2020).

In *Aedes* mosquitoes, mutations in eye-color genes, such as the *red-eye* and *white-eye* genes, have been used to construct different GSSs in *Ae. aegypti* through classical genetics (Koskinioti *et al.*, 2021). These selectable markers are particularly powerful because they are stable, the phenotype is evident from the first instar larvae until the adult stage, and they are located on chromosome 1, linked to the male-determining region (Bhalla & Craig, 1970; Munstermann & Craig, 1979). The red-eye GSS has demonstrated better quality and genetic stability than the white-eye GSS, and its sexing properties have been successfully transferred to different genetic backgrounds (Augustinos *et al.*, 2022; Misbah-ul-Haq *et al.*, 2022b). Employing marker-assisted mapping (MAM) of informative recombinants and analyzing the rate of homozygosity, the gene *cardinal* was identified as the cause of the red-eye trait in *Aedes* species (Carballar-Lejarazú *et al.*, 2020; Chen *et al.*, 2022). This marker was first reported nearly 60 years ago (McClelland, 1966). Representing a novel application of the bulk-segregant analysis (Schneeberger, 2014), MAM can be employed in identifying causal genes in genomic regions of suppressed recombination previously inaccessible (Chen *et al.*, 2022).

Quality control

Any material released into the environment, whether produced through classical, transgenic, or neoclassical genetic approaches, must be thoroughly evaluated before field application. This evaluation should consider their biological quality, including survival ability, food and water search efficiency, and importantly, their competitiveness with wild insects for mating. A significant amount of literature and experience from operational SIT projects worldwide has led to the creation of a manual that is

now utilized globally (FAO/IAEA/USDA, 2019). Parameters such as fertility, egg-to-pupae survival, pupal-to-adult recovery, and sex ratio should be measured. Another quality indicator is pupal weight, which correlates with the size of resulting GSS adult males. Larger adult insects are stronger, live longer, have higher mating propensity, and induce refractory periods in female flies compared to smaller males. However, testing the GSS quality under small-scale rearing conditions does not guarantee ideal performance in large-scale rearing conditions. Therefore, all the tests mentioned should also be conducted in semi-field conditions, including survival under stress and mating competitiveness tests. Flies must survive long enough, particularly under stress, to reach sexual maturity and seek mates. Sterile males should exhibit sexual competence to outcompete wild males for mating with wild females, thereby inducing sterility in the target population.

Another point for consideration is the development of behavioral resistance in wild-type females. Any genetic and/or phenotypic differences between GSS and wild insects raise the possibility of assortative mating. If wild females recognize differences between wild and mass-reared sterile males, selection in females may result in preferential mating with wild males. Behavioral resistance was reported during the eradication program of the melon fly *Z. cucurbitae* in Japan (Hibino & Iwahashi, 1991) and the population control project against *C. capitata* on Kauai island in Hawaii, USA (McInnis *et al.*, 1996). As behavioral resistance may occur in any GSS males, regardless of whether they have been developed through classical, transgenic, or neoclassical approaches, genetic refreshment of mass-reared colonies with wild material should be performed at regular intervals.

The genetic stability of the GSS should also be monitored over time to maintain its integrity and sexing properties. Instability is mainly attributed to genetic recombination. In GSS developed through classical genetic approaches, challenges can be overcome by selecting specific translocation lines (with reduced distance between the breakpoint and the marker), inducing inversions covering the region of the breakpoint and the marker, establishing a filter rearing system, or a combination of these methods (Caceres, 2002; Augustinos *et al.*, 2017; Franz *et al.*, 2021). It is also worth noting that recombination rates may vary significantly between males and females, depending on the species. For example, in *C. capitata*, there is a lower recombination rate in males than females. In *Aedes* mosquitoes, recombination events in males occur almost as frequently as in females. Although recombination is not expected to be a major issue in neoclassical GSS, if the rescue allele is closely linked to the male-

determining region, the establishment of a filter-rearing system may still be considered.

Regulatory framework for genetically modified insects

Countries have various regulatory frameworks for genetically modified insects, typically emphasizing environmental safety, public health, and ethics. Stringent evaluations often precede the release of such insects, especially for pest and disease vector control. Regulatory assessments focus on potential risks to biodiversity, human health, and ecosystems, while balancing these risks against benefits such as pest reduction and disease prevention.

In the United States, genetically modified insects used for population control are regulated as biopesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) by the Environmental Protection Agency (EPA). This regulatory framework includes transgenic insects aimed at controlling pest populations. The EPA evaluates potential risks to human health, non-target organisms, and environmental safety before authorizing their release (EPA, 2020; EPA, 2022) (<https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/epas-regulation-biotechnology-use-pest-management>). Trials and deployments must demonstrate a lack of harmful effects on non-target species and the ecosystem. Similarly, in Brazil, transgenic mosquitoes have been released under a regulatory framework governed by the country's National Biosafety Technical Commission (CTNBio) (Andrade *et al.*, 2018). These regulations focus on monitoring ecological impacts and human health risks, with controlled trials being a key part of the regulatory process (European Commission, 2021). In Australia, regulations on GMOs are coordinated by the Office of the Gene Technology Regulator (OGTR) (<https://www.ogtr.gov.au/about-ogtr/australias-gene-technology-regulatory-system>). According to the OGTR, modified organisms can be regulated as normal organisms if only knockouts are involved, whereas knock-ins are considered GMOs (Mallapaty, 2019; Wray-Cahen *et al.*, 2024).

The EU's regulatory stance on genetically modified organisms (GMOs) has long been critiqued for not keeping pace with technological advancements such as CRISPR/Cas9 and other gene-editing technologies. These tools enable precise and targeted changes in an organism's genome without introducing foreign DNA. However, the 2018 European Court of Justice ruling classified these gene-editing techniques as GMOs, mean-

ing they were subject to the same regulatory oversight as traditional genetically modified organisms, regardless of the technology used (European Court of Justice, 2018).

Recognizing the limitations of the current legislations, the European Commission proposed significant regulatory changes in 2023. The proposal aims to differentiate between two types of New Genomic Technologies (NGT) plants: (1) Category 1 NGT plants, which involve changes that could occur naturally or via conventional breeding, will be exempt from strict GMO regulations and will not require risk assessments. (2) Category 2 NGT plants, which include more complex genetic modifications, will still fall under the GMO framework but with simplified risk assessments and procedures (European Commission, 2023; Bohle *et al.*, 2024).

This shift is part of a broader effort to encourage innovation in agriculture and biotechnology while maintaining safety and environmental protection. The new regulation introduces risk-based rules that reflect the actual risk posed by each type of genetic modification, thus moving away from the precautionary principle that has historically slowed the adoption of NGTs in the EU (EASAC, 2024). The revised framework aligns with the goals of the European Green Deal (https://commission.europa.eu/strategy-and-policy/priorities-2019-2024/european-green-deal_en) and the Farm to Fork Strategy (https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en), promoting sustainable and resilient crop varieties.

While this proposal represents a major step forward for NGTs, debates continue about their coexistence with organic farming, the labeling of NGT products, and the regulation of NGTs in animals and microorganisms. As the European Parliament and Member States finalize the legislation, these aspects will likely influence the future adoption and impact of NGTs across Europe (Roger, 2023).

Conclusions

The development of efficient and genetically stable GSS is essential for enhancing the cost-effectiveness and scalability of the SIT for pest and vector control. While classical genetic approaches have been successfully implemented for certain species, their reliance on irradiation-induced chromosomal translocations and extensive screening makes them labor-intensive and time-consuming. Transgenic methods, although offering greater precision, face regulatory and societal constraints that limit their widespread use.

The *Neoclassical Genetic Approach*, based on CRISPR/Cas genome editing, presents a transformative solution by enabling the targeted introduction and linkage of sexing traits across multiple insect species. This approach combines the robustness of classical genetics with the precision of genome engineering, allowing for the rapid and stable development of GSS. It provides the flexibility to identify and modify conserved selectable markers, integrate them into sex-specific chromosomal regions, and minimize recombination-induced genetic instability. Moreover, neoclassical GSS development can also be beneficial for Incompatible Insect Technique (IIT) and combined SIT/IIT applications (Zheng *et al.*, 2019; Martin-Park *et al.*, 2022; Lim *et al.*, 2024), as an extremely strict sex separation system is required for successful IIT applications. Moreover, new SIT applications that incorporate insect growth regulators and biocides can also benefit from more stable GSS (Bouyer & Lefrançois, 2014; Herbillon *et al.*, 2024).

Future efforts should focus on refining CRISPR-based gene knock-in strategies, particularly in highly heterochromatic sex-determining regions, and assessing the long-term genetic stability and field performance of newly developed GSS. Advances in genomic sequencing, transcriptomics, and bioinformatics will be critical in overcoming current limitations, ensuring the broad applicability of neoclassical GSS across diverse pest species.

By integrating modern genome editing into established SIT frameworks, the *neoclassical genetic approach* offers a scalable, precise, and transferable method for the development of next generation of genetic sexing strains. This will strengthen global area-wide integrated pest management (AW-IPM) programs and contribute to more sustainable, effective insect population suppression strategies.

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Disclosure

The authors declare no conflicts of interest.

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