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Review

Biotechnology-enhanced genetic controls of the global pest *Drosophila suzukii*

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Spotted wing *Drosophila* (*Drosophila suzukii* Matsumura, or SWD), an insect pest of soft-skinned fruits native to East Asia, has rapidly spread worldwide in the past 15 years. Genetic controls such as sterile insect technique (SIT) have been considered for the environmentally friendly and cost-effective management of this pest. In this review, we provide the latest developments for the genetic control strategies of SWD, including sperm-marking strains, CRISPR-based sex-ratio distortion, neoclassical genetic sexing strains, transgenic sexing strains, a sex-sorting incompatible male system, precision-guided SIT, and gene drives based on synthetic *Maternal effect dominant embryonic arrest* (*Medea*) or homing CRISPR systems. These strategies could either enhance the efficacy of traditional SIT or serve as standalone methods for the sustainable control of SWD.

Highlights

Genetic control is a biological control method that introduces traits that sterilize, kill, or modify the population via intraspecific mating. Therefore, it is regarded as a species-specific and environmentally friendly management option for pest species.

Spotted wing *Drosophila* (SWD) is an ideal insect model for studying genetic control strategies due to its pest status, laboratory-friendly biology, and close relationship to *Drosophila melanogaster*, which has abundant genetic resources.

Different biotechnology-enhanced genetic control strategies of SWD are featured. The working schematics, control efficacies, some resistance mechanisms, and possible future development of these strategies are described.

The designs and experience from these studies aid in the sustainable control of SWD and serve as essential references to other insect pests of economic or public health importance.

Global invasion and management of spotted wing *Drosophila*

SWD (*Drosophila suzukii* Matsumura; Diptera, Drosophilidae), native to East Asia, has attracted attention from fruit growers and insect researchers over the past 15 years. SWD was not viewed as an important agricultural pest in the 20th century until it arrived in Western United States and Western Europe in 2008 [1,2]. The fly was rapidly transmitted to all major continents in the following years and caused considerable economic losses [3,4] (https://gd.eppo.int/taxon/DROSSU/distribution). The pest status of SWD is linked to its serrated ovipositor, which allows females to puncture the soft skin of ripening fruit [5,6]. Unlike most *Drosophila* species, which feed on rotting fruits, SWD females can penetrate the intact skin of fruit and lay their eggs (see Video S1 in the supplemental information online). The hatched larvae then move and feed inside the fruit, leading to rapid decay (see Video S2 in the supplemental information online). Such damage and possible secondary infestation by other insect pests or disease pathogens render the fruits unmarketable [5,7].

Over the past decade, considerable efforts have been made worldwide to prevent or control SWD. These efforts largely involved traditional components of **integrated pest management (IPM)** (see Glossary) [3,7–10]. Genetic methods such as **SIT**, a birth control strategy using radiation-sterilized insects, have also been considered for **area-wide IPM (AW-IPM)** programs for SWD (Figure 1A) [11,12]. Additionally, SIT has been combined with the **incompatible insect technique (IIT)**, another **genetic control** measure based on *Wolbachia* infection [13,14]. Such strategies are species-specific, because the control effects depend on intraspecific mating with a target population [15,16]. However, some challenges may affect the efficiency or implementation of SIT-like programs, such as reduced insect fitness due to mass rearing and/or radiation, decreased effectiveness because of mating between coreleased males and females, additional rearing costs and host plant damage from releasing ovipositing females, and inefficient monitoring due to the low persistence and detectability of insect marking techniques [15–17]. To address these challenges, various molecular systems have been used to create genetic control strains in different insect pests [15,16]. This review summarizes a variety of genetic control strategies that have been demonstrated in SWD or recently developed in other insect species, proposed for a

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robust control program of SWD (Figure 1). These strategies aim to either enhance the efficacy of traditional SIT or serve as standalone methods for pest control (Table 1).

Sperm-marking strains

Ensuring that released insects mate competitively under field conditions is crucial for SIT-like control programs. Specifically, the sperm produced by released males should be capable of inseminating wild-type (WT) females and remain competitive against sperm from WT males in case of remating. To monitor sperm transfer, storage, and competition, sperm-marking strains have been developed in SWD using the endogenous spermatogenesis-specific *β2-tubulin* (*β2t*) promoter to control the expression of a fluorescent protein gene (Figure 1B) [18,19]. In these sperm-marking strains, the *β2t* promoter induces testis-specific fluorescence from the third instar larval stage, enabling sex separation with 100% accuracy [19]. This sex separation can potentially be scaled up using high-throughput automated systems such as the Complex Object Parametric Analyzer and Sorter (COPAS), which has been used to efficiently sort fluorescent embryos of *Drosophila melanogaster* and sex fourth instar larvae of the mosquito *Anopheles stephensi* [20,21]. Notably, the fluorescent sperm can also be detected in the spermathecae of WT females, confirming successful mating between sperm-marked and WT flies [18,19]. The presence of such sperm can also be identified by PCR, which amplifies the DNA fragment of the marker gene [19]. Thus, sperm-marking strains provide visual and molecular tools to monitor insect mating within a release program.

CRISPR-based sex-ratio distortion

Besides regulating sex-specific fluorescent protein expression, the respective *β2t* promoter was also used to drive expression of the endonuclease **Cas protein 9 (Cas9)** in male germ cells when combined with a **guide RNA (gRNA)** targeting a repetitive DNA sequence encoding rRNA that is located exclusively on the X chromosome [22–24]. This approach, known as 'X shredding,' selectively destroys the X chromosome during spermatogenesis based on **CRISPR/Cas9**-induced mutagenesis, generating predominantly Y chromosome–bearing sperm and resulting in offspring with a strong male bias in sex ratio (Figure 1C). Such targeted mutagenesis works through insertions and deletions (indels), often caused by the **nonhomologous end joining (NHEJ)** repair pathway, at the double-strand breaks mediated by the CRISPR/Cas9 system.

The **CRISPR-based sex-ratio distortion (CRISPR^{SRD})** strategy was demonstrated in *Anopheles gambiae* and *Ceratitis capitata*, achieving up to a 94.8% and 80% male bias in these species, respectively [24,25]. The approach was also applied in *D. melanogaster* by targeting repeat sequences in the X-linked gene *Muc14a*, resulting in a 68% male bias [26]. Furthermore, targeting the putative haploinsufficient gene *Rps6*, also located on the X chromosome, led to a >92% male bias in the offspring by causing the death of most females at early developmental stages, a phenomenon referred to as 'X poisoning' because females die due to the heterozygous deficiency of the mutagenized X-linked gene [26]. It is important to note that X shredding is based on prezygotic effects, whereas X poisoning acts post-zygotically, leading to inviable female embryos. As a result, X poisoning could reduce reproductive output and might be less effective than X shredding for a control program [26,27]. Given the promising results in the mentioned insect species, CRISPR^{SRD} has been proposed for the control of SWD [28,29]. Implementing such strategies in SWD requires the identification of haploinsufficient gene loci or short, highly abundant sequence elements exclusively located on the X chromosome. A computational pipeline named 'Redkmer' was developed specifically for the CRISPR^{SRD} strategy to process whole-genome sequence data from males and females and predict candidate targets based on their X specificity and abundance [28]. Bioinformatic tools such as Redkmer are expected to aid in the development of CRISPR^{SRD} in SWD.

Glossary

Area-wide integrated pest management (AW-IPM): an effective and sustainable control approach that proactively targets entire pest populations.
Cas protein 9 (Cas9): a CRISPR-associated nuclease from *Streptococcus pyogenes* that can cleave DNA by forming site-specific double-stranded breaks.
CRISPR: (clustered regularly interspaced short palindromic repeats), a gene-editing technology that was originally derived from the adaptive immune system of bacteria and archaea.
CRISPR-based sex-ratio distortion (CRISPR^{SRD}): a genetic control strategy that selectively destroys the X chromosome during spermatogenesis using the CRISPR system. Therefore, it generates offspring with a strong male bias in sex ratio.
Female killing (FK): a genetic control strategy that selectively kills females.
Female-specific (fs) splicing element: a female-specific intron from the sex determination gene *transformer* in *Ceratitis capitata* (*CctraF*) or *Cochliomyia hominivorax* (*ChtraF*).
Genetic control: also known as 'genetic pest management' (GPM), is a biological control method that introduces traits that sterilize, kill, or modify the population via intraspecific mating.
Gene drives: methods for promoting the spread of specific genes through a population by ensuring they are inherited more frequently than through traditional Mendelian inheritance.
Guide RNA (gRNA): a single chimeric RNA that guides the Cas protein to its target sequence.
Genetic sexing strains (GSSs): insect strains with sex-specific phenotype(s) that allow efficient sex separation for SIT application. The sex-specific phenotype (s) are typically due to naturally occurring mutation or mutation generated by classical genetic approaches.
Homing endonuclease genes (HEGs): selfish genetic elements that encode endonucleases recognizing a specific sequence that flanks the HEG. Therefore, they can spread by first cleaving chromosomes that do not contain HEG and then copied across to the cut homolog, using themselves as a template for the repair process.
Homology-directed repair (HDR): one of the two repair pathways for CRISPR-induced double-stranded breaks. It can

Neoclassical genetic sexing strains

Genetic sexing strains (GSSs) have been a cornerstone in SIT programs for decades [17,30]. Recently, **'neoclassical' GSSs** have been proposed using the CRISPR system in a more convenient, flexible, and transferable manner (Figure 1D and Box 1). A specific example for SWD is the creation of the *temperature-sensitive* (ts) mutation in the sex determination gene *transformer 2* (*tra2*) through CRISPR-induced **homology-directed repair (HDR)**, another repair pathway for double-strand breaks [31]. This $tra2^{ts2}$ mutation is a known ts allele in *D. melanogaster* [32]. Maintaining SWD $tra2^{ts2}$ flies at temperatures below 20°C resulted in normal fertile XX and XY adults. By comparison, exposure to restrictive temperatures (26°C to 29°C) produced non-mating sterile XX pseudomales and sterile XY males, which could mate with WT females but produced no offspring [31], mirroring the behavior expected in *D. melanogaster* XY *tra2*-mutant males [32]. This approach offers a simple method for achieving sex selection and male sterility by manipulating rearing temperatures. However, both $tra2^{ts2}$ XX and XY flies exhibited reduced viability at restrictive temperatures, potentially limiting their application in control programs. Nonetheless, this research demonstrates in SWD that a point mutation can be precisely engineered toward a neoclassical GSS using the CRISPR system.

Transgenic sexing strains

Transgenic sexing strains (TSSs) have been developed for various agricultural pests and vectors of human diseases [15,33]. TSSs typically use the food additive controlled binary **tetracycline-off (Tet-off) system** [34], which is composed of the ***tetracycline transactivator* (*tTA*)** and the ***tetracycline response element* (*TRE*)** driving a gene causing lethality [35,36]. By employing the Tet-off system to conditionally express an effector lethal gene only in females, TSS eliminates all females from the population to be released by feeding the insects a diet devoid of tetracycline [35,36]. This approach, also referred to as '**female killing**' **(FK)**, is considered potentially more efficient than traditional SIT if the FK effect is dominant [27,37]. To further decrease the costs associated with mass rearing, **transgenic embryonic sexing systems (TESSs)** have been introduced for several tephritid fruit flies and livestock pests, targeting the elimination of females at the embryonic stage (Box 2). Several FK strains based on TESS have been generated in SWD by engineering all components into one *piggyBac* vector in an all-in-one strategy, which exhibited up to 100% female lethality in the absence of tetracycline [38]. However, the FK strains produced so far are not functional for a control program, because **true-breeding lines** could not be generated due to the leaky lethality of homozygous females with tetracycline food. Meanwhile, varying concentrations and derivatives of antibiotics (tetracycline or doxycycline) exhibited parental and transgenerational effects on the lethality and fitness of a selected FK strain [39], emphasizing the need for cautious use of antibiotics in rearing insect strains engineered with the Tet-off system.

One strategy in pursuit of advanced TSS involves an autoregulation loop of *tTA* by placing *tTA* under the direct control of the *TRE* [40]. This autoregulation leads to very high levels of *tTA*, which results in insect mortality, presumably due to 'transcriptional squelching' or inhibition of ubiquitin-mediated proteolysis [41]. This system was further advanced in SWD by adding to the autoregulation of tTA a proapoptotic gene controlled by the same TRE (transgene named FL19). The bidirectional design of this system allowed the expression of two lethal effector genes in females, leading to the production of a male-only population in the absence of tetracycline (Figure 1E) [42]. Strains could be obtained carrying FL19 homozygously, in which the dominant female lethality could still be efficiently counteracted by adding tetracycline to the diet. Male inheritance of the dominant female lethality could collapse SWD populations within 8 weeks [42] in laboratory cage trials with repeated releases of FL19 males at excessive numbers (with initial FL19 male:WT male ratios of 10:1 or 13:1). This indicates that TSS might also be used for SIT approaches without irradiation. Should the first obtained transgenic lines not show the expected characteristics, remobilization of the transgenes to other genomic positions by employing

generate specific mutations or introduce genes of interest into the genome by providing the corresponding DNA template for the repair.

Incompatible insect technique (IIT): a pest control strategy in which insects are infected with *Wolbachia* and released into the field; mating between the infected male and a wild-type female produces no offspring due to cytoplasmic incompatibility.

Integrated pest management (IPM): a control approach that relies on a combination of harm-reducing practices.

Maternal effect dominant embryonic arrest (*Medea*): a genetic system that was first discovered in the flour beetle, in which a region of nuclear DNA (known as the *Medea* gene) causes the death of all offspring of heterozygous females that do not inherit a copy of the *Medea* gene.

Neoclassical genetic sexing strains: GSSs generated using no transgenic material and certain gene editing technologies, such as the CRISPR system, to induce the phenotype- and the sex-specific rescues.

Nonhomologous end joining (NHEJ): one of the two repair pathways for CRISPR-induced double-stranded breaks, which causes random insertions or deletions at the targeted site.

***piggyBac* jumpstarter line:** a transgenic line that expresses the *piggyBac* transposase therefore can be used to remobilize the transgene containing *piggyBac* 3′ and 5′ ends within the host genome.

Precision-guided sterile insect technique (pgSIT): a genetic control strategy that aims to achieve 100% female lethality and 100% male sterility through CRISPR-mediated gene targeting.

Tetracycline-off (Tet-off) system: a binary molecular system that allows conditional gene expression by adding or removing tetracycline.

***Tetracycline response element* (*TRE*):** the tTA binding site that consists of multiple tetracycline operator sequences.

***Tetracycline transactivator* (*tTA*):** a fusion of the DNA binding domain of the *E. coli* tetracycline repressor and the transcription activation domain from the HSV-1 tegument protein VP16.

Transgenic embryonic sexing system (TESS): a genetic control strategy that employs transgene(s) to conditionally eliminate females at the embryonic stage.

***piggyBac* jumpstarter lines** might improve the performance [43,44]. This approach produced several lines harboring two copies of FL19 on chromosome 3, exhibiting promising fitness parameters for SIT [45]. Consequently, these TSSs hold potential for further evaluation toward field application.

Transgenic sexing strains (TSSs): insect strains with sex-specific phenotype(s) that is conferred by the transgene(s).
True-breeding line: an insect line that is homozygous for a particular trait and can be self-sustained without losing this trait.

Sex-sorting incompatible male system

By combining a female-specific lethality system based on tTA autoregulation with an engineered genetic incompatibility (EGI) system that causes hybrid lethality, a sexing strain can be established that does not need irradiation, and the respective system has been termed the 'sex-sorting

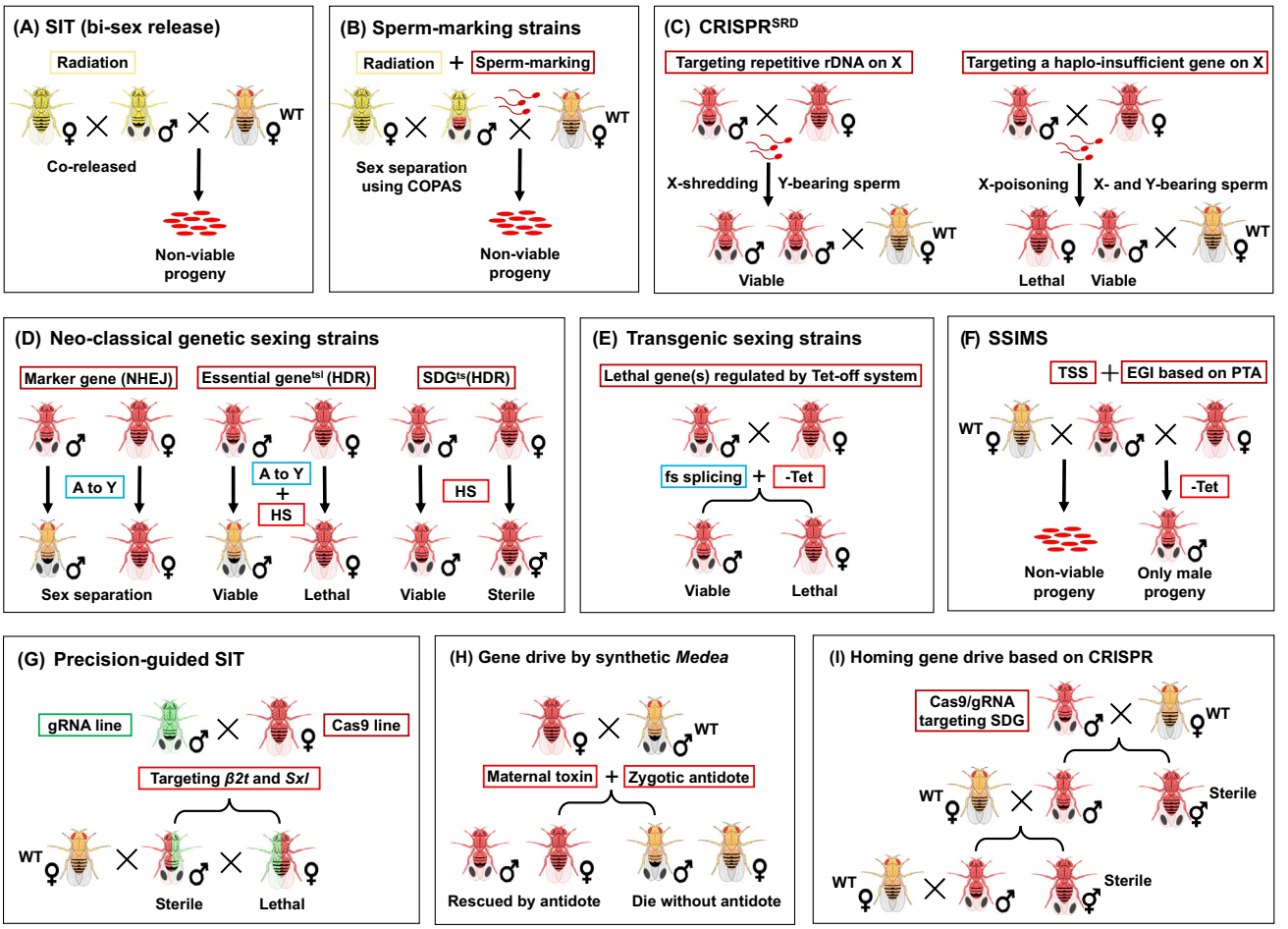

Figure 1. Genetic control strategies for spotted wing *Drosophila* (SWD). (A) Sterile insect technique (SIT): releasing radiation-sterilized insects produces nonviable offspring when they mate with wild-type (WT) females. (B) Sperm-marking strains: expressing fluorescent markers in testis and sperm, such strains could facilitate sex separation using the Complex Object Parametric Analyzer and Sorter (COPAS) and mating monitoring. (C) CRISPR-based sex-ratio distortion (CRISPR^{SRD}): targeting X chromosome-specific rDNA (most zygotes develop into males due to X shredding) or a haploinsufficient gene *Rps6* (nearly half of zygotes die of X poisoning) by the CRISPR system, leads to male-dominant populations. (D) Neoclassical genetic sexing strains (GSSs): relying on CRISPR-induced mutations in marker, essential, or sex determination genes (SDGs) that enable sex-specific phenotypes for separation. (E) Transgenic sexing strains (TSSs): leveraging the Tet-off system to express lethal genes with a **female-specific (fs)** intron, this approach could result in male-only populations for release. (F) Sex-sorting incompatible male system (SSIMS): integrating TSS with engineered genetic incompatibility (EGI) based on the programmable transcriptional activator (PTA), this system produces males that are incompatible with WT females. (G) Precision-guided sterile insect technique (pgSIT): crossing a homozygous line expressing Cas9 with a homozygous line expressing guide (g)RNAs targeting the *β2t* gene for male sterility and *Sxl* for female lethality. (H) Synthetic *Maternal effect dominant embryonic arrest (Medea)* system: consisting of linked 'toxin' and 'antidote' genetic cassettes, this system mimics natural *Medea* and promotes the spread of linked genes due to the survival advantage of *Medea* carriers. (I) CRISPR-based homing gene drive: targeting SDG for sterile, intersex phenotypes could facilitate population suppression by promoting super-Mendelian inheritance. Abbreviations: HDR, homology-directed repair; HS, heat-shock; NHEJ, nonhomologous end joining; Tet, tetracycline.

Table 1. Genetic control strategies that were demonstrated or proposed for SWD

Strategy	Working mechanism[a]	Effector/target gene[b]	Functional phenotype(s)	Developmental status	Refs
Sperm marking	Fluorescent gene regulated by spermatogenesis-specific promoter	*β2t_DsRed*	Testis- and sperm-specific expression	Illustrated in laboratory for *Drosophila suzukii*	[18]
		β2t_AmCyan			[19]
CRISPR^{SRD}	CRISPR-induced mutagenesis	*muc14a* or *RpS6*	X chromosome shredding (*Muc14a*) or poisoning (*RpS6*)	Demonstrated in *Drosophila melanogaster* and proposed for SWD	[26,28,29]
Neoclassical GSS	CRISPR-induced HDR	*tra2*	Sex conversion	Illustrated in laboratory for SWD	[31]
	CRISPR-induced mutagenesis	*wp*	White pupae	Demonstrated in *Ceratitis capitata* and *Bactrocera tryoni* and proposed for SWD	[85]
TSS	Conditional lethality based on Tet-off system	*Dshid^{Ala4}_CctraF*	Dominant female lethality	Illustrated in laboratory for SWD	[38]
		tTA_ChtraF + *Dmhid^{Ala5}* or *Dsrpr*	Dominant female lethality	Demonstrated in cage experiments for SWD	[42]
SSIMS	Conditional lethality based on Tet-off system and EGI	*tTA_ChtraF* + *pyramus* regulated by *dCas9-VPR*	Dominant female lethality and male genetic incompatibility	Demonstrated in *D. melanogaster* and agent-based modeling in SWD	[46,47]
Precision-guided SIT	CRISPR-induced mutagenesis	*Sxl* (*tra*, *dsx*) + *β2t*	Female lethality (sex conversion) and male sterility	Demonstrated in *D. melanogaster* and illustrated in cage experiments for SWD	[49,50]
Gene drive	*Medea*	*myd88*	Embryonic lethality	Demonstrated in cage experiments for SWD	[69]
	CRISPR-mediated HDR	*tra*	Sex conversion and sterility	Demonstrated in *D. melanogaster* and proposed for SWD	[96]
	CRISPR-mediated HDR	*dsx*	Sex conversion and sterility	Demonstrated in SWD	[71]

[a]Genetic systems that were employed: HDR (homology direct repair), Tet-Off (tetracycline-Off), EGI (engineeredd genetic incompatibility), *Medea* (*maternal effect dominant embryonic arrest*).
[b]Targeted or effector genes include *DsRed* gene regulated by *β2-tubulin* gene promoter (*β2t_DsRed*), *AmCyan* gene regulated by *β2-tubulin* gene promoter (*β2t_AmCyan*), *myeloid differentiation factor 88* (*myd88*), *transformer* (*tra*), *Sex-lethal* (*Sxl*), *β2-tubulin* (*β2t*), *doublesex* (*dsx*), *Mucin 14A* (*muc14a*), *Ribosomal protein S6* (*RpS6*), a phosphomutated version of SWD *head involution defective* gene containing a female-specific intron from *C. capitata tra* gene (*Dshid^{Ala4}_CctraF*), *tetracycline transactivator* containing a female-specific intron from *C. hominivorax tra* gene (*tTA_ChtraF*), a phosphomutated version of *D. melanogaster hid* (*Dmhid^{Ala5}*), *D. suzukii reaper* gene (*Dsrpr*), *pyramus* gene regulated by dCas9-VPR (VP64-p65-Rta) which is a PTA.

incompatible male system' (SSIMS; Figure 1F) [46,47]. EGI acts similarly to post-zygotic speciation mechanisms and uses a dCas9-based **programmable transcriptional activator (PTA)** that activates a gene that is lethal when overexpressed or ectopically expressed. The lethality is avoided in the EGI line by making it homozygous for a mutation that prevents binding of the

Box 1. GSS in Medfly

A classical GSS in the Mediterranean fruit fly (Medfly) uses two naturally occurring recessive loss-of-function mutations: *white pupae* (*wp*) and *temperature-sensitive lethal* (*tsl*). Following radiation exposure, Y chromosomes containing translocated WT alleles of these two genes were recovered. As a result, females homozygous for the mutations display white puparia and die at the embryonic stage when exposed to elevated temperatures (34–35°C) for 24 h. Males, convesely, exhibit brown puparia and survive under the same elevated temperatures [30,84]. For the neoclassical GSS, it is proposed that CRISPR-based gene editing technologies can facilitate the creation of desired mutations. For instance, CRISPR-induced mutagenesis has been employed to induce loss-of-function mutations in the phenotypic marker gene *wp* in Medfly and other tephritid fruit flies [85]. Additionally, CRISPR-induced translocations might be applied to insert the respective WT alleles into the Y chromosome [86,87]. Classical mutagenesis studies in *D. melanogaster* offer critical insights for developing such 'neoclassical' GSSs. For instance, a compilation of essential genes from *D. melanogaster* with verified recessive or dominant temperature-sensitive (ts) phenotypes has been suggested as potential CRISPR targets for generating ts-based GSSs [88].

Box 2. TESS in dipteran pests

TESS typically involves the combination of a driver strain, which carries a transgene where the *tetracycline transactivator* (*tTA*) is under the control of a cellularization gene promoter, and an effector strain, which harbors a transgene in which a lethal gene is linked to the *tetracycline response element* (*TRE*) consisting of multiple *tetO* operator sequences [43,89–93]. The lethal gene adjacent to *TRE* incorporates a female-specific intron from the sex determination gene *tra* (*traF*), ensuring its expression exclusively in females in the absence of tetracycline. TESSs for tephritid flies, including *C. capitata*, *Anastrepha suspensa*, and *Anastrepha ludens*, have employed the *traF* from *C. capitata* (*CctraF*) [43,89,91], whereas TESSs for livestock pests such as *Lucilia cuprina* and *Cochliomyia hominivorax* have used the *traF* from *C. hominivorax* (*ChtraF*) [90,92,93]. Additionally, the driver and effector cassettes have been consolidated into a single transgene for an all-in-one (AIO) strategy, which can be integrated at multiple loci for enhanced suppression effects [37,90].

To develop the TESS strategy in SWD, four cellularization gene promoters (*nullo*, *serendipity-α*, *bottleneck*, and *slow-as-molasses*) and three proapoptotic genes (*head involution defective* or *hid*, *grim*, or *reaper*) were isolated from this pest and evaluated *in vitro* [94,95]. Those demonstrating promising promoter activities or lethal effects were engineered into *piggyBac* vectors in an AIO manner [38]. These constructs used the female-specific intron from the *Cctra* gene to restrict the expression of the proapoptotic protein to females. SWD strains transformed with an AIO construct, containing *Dsnullo*-regulated *tTA* and a phosphomutated version of *hid* (*Dshid*^{Ala4}), exhibited up to 100% female lethality in the absence of tetracycline. However, females from these strains died at later developmental stages rather than early ones, and homozygous females also perished even when fed tetracycline-supplemented food, indicating that basal expression of the TRE-linked proapoptotic gene is lethal. Thus, the TESS for SWD requires refinement to ensure that lethality is early stage, female specific, and fully conditional.

PTA and, thus, overexpression. Outcrossing with WT produces inviable hybrid offspring, because they inherit a copy of the PTA that causes lethal overexpression or ectopic expression of the targeted gene from the WT allele.

Leveraging the *tTA*-induced female lethality and PTA-mediated reproductive barrier, the SSIMS line eliminates all females at larval/pupal stages in the absence of tetracycline, producing males that are genetically incompatible with WT females, since such crosses produce inviable offspring, making them suitable for release in a SIT approach without irradiation. Cage trials revealed that releasing SSIMS males at a 1:1 ratio with WT flies effectively suppressed the WT population within 10 weeks [46]. Agent-based modeling indicated that SSIMS could achieve SWD population suppression in a single growing season, with its efficacy depending on the tetracycline concentrations provided to the released flies, thus controlling female lethality effects on the released or descendant generations [46]. Considering endemic genetic resistance, where resistance alleles in the wild population might lead to EGI survivors, modeling suggested that a dual-EGI strategy or a linked SSIMS, wherein the EGI and female lethality transgenes are genetically linked, could effectively counteract resistance [47]. Another bioinformatic pipeline, haplotype-resolution analysis of unique loci by bulk genomic extraction (HUGE), was developed to feature the genetic variation within SWD field populations [48]. This tool can be used to evaluate the control efficacy of SSIMS strategies by detecting SNP frequencies at genomic locations that are targeted by PTA.

Precision-guided SIT

Another approach for eliminating females and producing reproductive sterile males has been termed '**precision-guided SIT**' **(pgSIT),** which was functionally demonstrated in *D. melanogaster* and has been transferred to SWD (Figure 1G) [49,50]. To achieve the desired phenotypes, genes involved in dosage compensation and sperm development, *Sex-lethal* (*Sxl*) and *β2t*, respectively, are targeted by CRISPR/Cas9-induced mutagenesis to cause female lethality and male sterility. Specifically, Cas9-expressing lines, where a germline or constitutive promoter regulates the Cas9 gene, and gRNA-expressing lines targeting the *Sxl* and *β2t* genes, were generated in SWD and maintained separately. In *D. melanogaster*, *Sxl* governs both sex determination and X chromosome dosage compensation, and its loss-of-function mutations result in the death of all XX individuals due to X chromosome hyperactivation [49,51,52]. Meanwhile, the *β2t* gene is involved in the development of sperm microtubules, and its loss-of-function mutations lead to defective sperm with reduced motility, causing male sterility

[49,53,54]. By crossing the Cas9-expressing line with the gRNA-expressing line targeting *Sxl* and *β2t* in SWD, 100% female lethality and 100% male sterility were achieved in the offspring [50]. Cage experiments demonstrated that repeated releases of pgSIT flies with maternal Cas9 (at a ratio of 5:1) led to population collapse within five generations [50], indicating the robustness of the pgSIT strategy for SWD control.

The current pgSIT approach for SWD necessitates maintaining both Cas9 and gRNA lines separately, because combining them into a single strain would result in lethal/sterile phenotypes [49,50]. Producing sterile males for release requires sexing and crossing these lines, which would increase operational costs in a control program. Temperature-inducible systems, using *hsp70* promoters to regulate the Cas9 gene, have facilitated CRISPR-mediated gene targeting in SWD [55], making it possible to create a true-breeding line containing both Cas9 and gRNA cassettes/transgenes under permissive temperature conditions. This was achieved in *D. melanogaster*, where a double-homozygous line containing both *Dmhsp70-Cas9* and gRNA transgenes targeting *tra* and *β2t* was maintained at 18°C. Upon switching the rearing condition to 26°C with additional heat shock treatment (1 or 2 h at 37°C), all females/intersexes from this line died, and all males were sterile [56]. Other gene regulation approaches, such as the Tet-off and quinic acid systems, could also enable the conditional generation of lethal/sterile phenotypes [34,57,58]. Additionally, a recent study identified two spermatogenesis-specific genes, *wampa* and *Prosα6T*, in SWD, which could be targeted for male sterilization strategies [59].

Gene drives

Gene drives have been proposed as a strategy for pest control for decades [60–62]. The discovery and application of novel genetic elements or systems have significantly advanced the development of gene drive strategies in insect pests, as recently reviewed [16,63–66]. Most of these developments were demonstrated in *D. melanogaster*, whereas gene drives based on selfish genetic elements including **maternal effect dominant embryonic arrest (*Medea*)** and CRISPR-designed **homing endonuclease genes (HEGs)** have been showcased in SWD (Figure 1H,I). Resistance mechanisms have been identified in these studies (Box 3), emphasizing

Box 3. Resistance to gene drives in SWD

In the synthetic *Medea* system, only one of four miRNA target sites remained 100% conserved among resistant individuals, with mutations identified in the other three target sites [69]. Notably, a deletion of an adenosine (A) within the vicinity of an miRNA seed sequence emerged as a particularly effective resistance allele, consistently found in populations with inheritance rates below 100%, even across geographically distinct populations [69]. Thus, selecting well-conserved target sites across different geographical populations and multiplexing miRNAs to enhance toxin efficiency may help mitigate the development of resistance. Moreover, long-term cage experiments demonstrated that the efficacy of gene drive systems can be influenced by initial introduction frequencies, particularly if preexisting resistance mechanisms are present. Specifically, the *Medea* frequency was not sustained when introduction frequencies were below 50%, likely due to drive resistance and the high fitness costs associated with the transgene, whereas it remained at high frequencies in populations with introduction frequencies over 90% [69]. Therefore, employing high introduction frequencies and/or multiple successive releases could potentially improve the performance of the *Medea* system [69].

For the *tra*-targeting homing gene drive in *D. melanogaster* as a reference to SWD, inframe indel mutations created by the NHEJ repair pathway were observed in resistant individuals, rendering the target site insensitive to the gRNA and stopping the drive [96]. These drive-resistant alleles appeared at relatively high ratios (up to 10% of all progeny), indicating that population resistance could develop quickly and compromise the initial gene drive design. Modeling suggested that employing multiple gRNAs could significantly reduce the emergence of inframe resistant alleles, with their formation rate decreasing exponentially with each additional gRNA, and that a population collapse could be achieved with a strategy combining multiple gRNAs and multiple successive releases [96]. For the *dsx*-targeting split gene drive in SWD, flies carrying small inframe deletions in the *dsx* female exon that could confer resistance were identified. Although the mutant versions of the *dsx* exon were shown to be resistant to Cas9 cleavage *in vitro*, *in vivo* analysis of these inframe *dsx* mutant alleles indicated they were not functional and would likely be lost from the population [71]. Nonetheless, it is conceivable that some *dsx* mutant alleles could remain functional, potentially rescuing female fertility and leading to population resistance against the drive.

the need for careful evaluation and potential improvement of the existing systems for the efficient control of SWD.

Synthetic *Medea* systems have been developed in *D. melanogaster* by targeting an essential embryonic gene, *myd88*, by maternally providing artificial miRNAs (toxin) and employing a zygotic rescuing transgene (antidote) [67,68]. This strategy was subsequently adapted for SWD by creating a transgenic line equipped with both a 'toxin' and an 'antidote' cassette [69]. The toxin cassette employs a maternal promoter (*bicoid*) to regulate four synthetic miRNAs that target the 5′ untranslated region (UTR) of *myd88*. By contrast, the antidote cassette uses a zygotic promoter (*bottleneck*) to regulate a recoded version of *myd88*, which is not recognized by the miRNAs. The miRNA toxin disrupts the maternal deposition of *myd88* mRNAs into the embryo, leading to the death of the progeny at the embryonic stage. However, for progeny that inherit a copy of the *Medea* gene, containing the resistant *myd88* version, which is expressed zygotic during early embryogenesis, rescues them from the lethal effect caused by a lack of maternal *myd88*. Consequently, in matings between heterozygous *Medea* females and WT males, half of the progeny that do not inherit *Medea* would perish, whereas the other half inheriting one copy would survive (selfish effect). Additionally, matings between heterozygous males and females would result in 75% viable progeny, because they could inherit one copy of *Medea* from either parent. The effectiveness of this synthetic *Medea* system was empirically validated in SWD, with the percentage of *Medea*-bearing progeny reaching up to 100% from heterozygous female outcrosses, rather than the 50% expected from standard Mendelian inheritance [69]. Thus, this system offers a potential strategy to disseminate desirable genes/phenotypes for controlling SWD populations.

For CRISPR-mediated homing gene drive, expression of Cas9 and a gRNA that were inserted into a target site in the genome results in the cleavage of the homologous chromosome at the same target site position. This DNA cleavage can be repaired via HDR, using the chromosome with the inserted Cas9-gRNA gene cassette as a template, thereby generating a second copy in the homologous chromosome, making the homing locus homozygous [62–64]. This homing process can thus convert a heterozygous genotype into a homozygous one, as first demonstrated in *D. melanogaster* [70]. For population suppression, the sex determination genes *tra* and *doublesex* (*dsx*) were targeted by a CRISPR-mediated homing drive for sex conversion effects in *D. melanogaster* and SWD, respectively. By inserting a Cas9-gRNA homing cassette into the first exon of the *D. melanogaster tra* gene and crossing such heterozygous males with WT females, up to 92% of the offspring carried the transgene, exhibiting super-Mendelian inheritance. Among the transgenic offspring, up to 96% were either phenotypic males or intersexes, confirming the sex conversion effects. In SWD, homing strains targeting the female exon of *dsx* were generated and crossed with Cas9-expressing strains for a split gene drive [71]. Offspring carrying both Cas9 and *dsx*-targeting gRNA were further crossed with WT flies, observing a range of 56–99% homing drive inheritances in the next generation. Notably, the Cas9-expressing strain, using the SWD *nos* promoter and 3′ UTR along with two SV40 nuclear localization signal (NLS) sequences for *Cas9* regulation, significantly and consistently promoted homing drive efficiency. Females with disrupted *dsx* were sterile due to a deformed ovipositor. Modeling showed that such a *dsx*-targeting gene drive could lead to the extinction of cage populations within ten generations [71].

Concluding remarks

Recent years have seen significant advancements in pest control strategies enhanced by genetic engineering and synthetic biology [15,16,72,73]. The improvement in sequencing technologies and bioinformatics also greatly aided in obtaining and sharing high-quality genomic and transcriptomic data in SWD [74–76]. In addition, some important genetic manipulation tools are demonstrated in

Outstanding questions

Besides the neoclassical GSS, all other strategies mentioned here will leave designed transgene(s) integrated into the insect genome. Strategies such as pgSIT and SSIMS need two independent transgenes to be functional. How would the potential fitness cost of the transgene(s) affect the mass rearing and field performance of the transgenic insect strain? How can genomic site(s) be selected for the transgene(s) with minimal fitness cost?

Spontaneous mutations in the integrated transgene could completely abolish the functionality of the control strategy. How could the rate of spontaneous mutations be efficiently determined for the target species? Would such rates differ under laboratory, mass rearing, and field conditions? How would such rates affect the implementation of the control program at large scales?

Strategies such as pgSIT and gene drives rely on Cas9-endonuclease and a gRNA targeting a precise gene sequence. Although designing and testing these strategies on the basis of sequence data from an inbred laboratory strain serves as a useful proof of concept, genetic polymorphisms within the target site could reduce the efficacy of such strategies by facilitating the selection and accumulation of resistant individuals. Therefore, how can population genetic variation be effectively discovered and quantified? How could specific gene targets be tailored to wild populations?

What are the technical obstacles to move these strategies from laboratory to field? For example, strategies such as TSS and SSIMS need food supplements (tetracycline) to rear the insect, and how would such supplements affect the management costs and the insect performance? Strategies such as sperm-marking strains and pgSIT would benefit from efficient sexing methods. How can sexing methods that are compatible with these strategies be developed? How can true-breeding lines for strategies conferring insect sterility, lethality, or sex distortion be generated, which are essential for mass rearing?

SWD with relatively high efficiencies, including transposon-mediated transformation and remobilization [44,77], recombinase-mediated genome targeting [78,79], and Cas9-expressing strains for CRISPR-mediated gene editing [55,80]. There are also resources and lessons from *D. melanogaster* that greatly facilitated the genetic studies in SWD (Box 4). All these resources, tools, and experiences allow researchers to develop and evaluate different genetic control strategies in SWD faster than ever before. Furthermore, there are some potential aspects that may limit the application of the introduced strategies (see Outstanding questions). For example, the verified spontaneous mutations shut off two functional transgenes, *tTA* and hid^{Ala5}, in a *D. melanogaster* lethal strain, generating survivors at a 5.8×10^{-6} frequency [81]. Therefore, evaluating the impacts of such spontaneous mutations on the performance of the transgene-based strategies is crucial if they are to be scaled up for mass rearing and releasing programs.

From the application perspective, genes encoding fluorescent proteins often serving as transformation markers can also help identify released insects. For strategies without a visible transgene marker, such as some neoclassical genetic sexing strains, fluorescent dusts may be required for marking released insects [82]. Certain approaches, including sperm-marking strains with COPAS and neoclassical GSS, enable efficient sexing to produce primarily or exclusively fertile males, which must be sterilized by radiation before release. Strategies such as $CRISPR^{SRD}$ and TSS can produce predominantly male populations suitable for both sterile or fertile releases if the FL or SRD effects are dominant. Modeling has indicated that fertile releases might be more effective than sterile ones, because FL or SRD alleles can be transmitted across generations, maintaining control effects in the field [27,37,83]. Strategies such as SSIMS and pgSIT are designed to eliminate females and produce males that are either sterile or incompatible with WT females, allowing these males to be released without the need for radiation. For gene drive strategies that leverage super-Mendelian inheritance, fertile releases are essential to facilitate the spread of desired genes/phenotypes within targeted populations.

The selection and implementation of specific genetic control strategies for SWD will vary on the basis of management objectives, such as geographical containment, prevention, population suppression, or eradication in targeted areas. Furthermore, rigorous risk assessments are crucial prior to field application to understand the potential impacts of control agents on ecosystems and human health. With its high-quality genomic resources, various genetic manipulation tools,

Box 4. Resources and lessons from *D. melanogaster*

The development of genetic control strategies in SWD benefits significantly from genetic resources and studies in *D. melanogaster*, including developmental/sex-specific/tissue-specific transcriptomes [97–99], genome-wide RNAi screens [100–102], and extensive mutagenesis studies. These serve as valuable references for designing genetic controls in SWD, such as identifying functional regulatory elements or suitable target genes. Molecular elements from *D. melanogaster*, such as the *PUb* promoter for marker gene expression [77,103], *hsp70* promoter for inducible expression [55], *nos* and *vasa* promoters for germline-specific expression [80], U6 gene promoter for gRNA expression [80,104], and the *hid* gene for induced lethality [42], have been directly used in SWD, demonstrating satisfactory activities. Meanwhile, some strategies were first demonstrated in *D. melanogaster* and then realized for SWD such as pgSIT and gene drives (see Table 1 in main text). However, findings from *D. melanogaster* do not always translate directly to SWD. For instance, SWD showed less sensitivity to the loss of TRA-2 activity than *D. melanogaster*, with SWD XY males carrying the $tra2^{ts2}$ mutation becoming sterile at 26°C, whereas *D. melanogaster* males with the same mutation did so at 18°C [31,32]. This indicates that neoclassical GSSs in SWD may react differently to temperature conditions during mass rearing and field release. Additionally, TSS of *D. melanogaster* based on tTA-overexpression toxicity killed 100% of female homozygotes without tetracycline [40], whereas TSS of SWD relying on overexpression of both *tTA* and the endogenous lethal gene *rpr* resulted in 98% female homozygote mortality under the same conditions [42], indicating the importance of screening enough independent transgenic lines to find the right combination for causing 100% lethality. Moreover, the *white* (w^-) strains of *D. melanogaster* that can be used for transgenesis generally exhibited reasonable fitness [105], whereas w^- strains of SWD obtained in different labs were largely sterile [80,104,106]. These differences highlight the need for case-by-case evaluation of gene phenotypes, molecular element performance, and the efficacy of molecular systems when developing genetic controls for SWD.

close relationship to the model organism *D. melanogaster*, developmental biology conducive to laboratory research and mass-rearing, and status as a global pest, SWD is an exemplary subject for developing and testing various genetic control strategies. Advances or future enhancements in SWD control strategies may also offer insights applicable to managing other economically or public health-significant insect pests.

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Declaration of interests

The authors declare no competing interests.

Supplemental information

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