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Advancing the sterile insect technique for the New World screwworm fly: the case for CRISPR-engineered genetic sexing strains

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The sterile insect technique (SIT) has long been a cornerstone of area-wide integrated pest management programs, and the eradication of the New World screwworm *Cochliomyia hominivorax* (Coquerel) from North and Central America is widely regarded as a landmark success. However, its resurgence underscores the need to modernize SIT strategies against this devastating pest. The development of genetic sexing strains (GSS) is a revolutionary idea supporting SIT, enabling efficient large-scale separation and the exclusive release of sterile males. Despite GSSs having been shown to significantly enhance the cost-effectiveness and field efficacy of operational SIT campaigns, no such sexing systems have yet been developed for *C. hominivorax*. Given recent advances in insect genomics, we consider the development of a screwworm GSS both feasible and timely. In this forum, we critically evaluate the potential for translating decades of GSS research into *C. hominivorax* by leveraging the CRISPR/Cas9 gene-editing technology. To make evidence accessible to a broader audience, we present a narrative overview covering the (i) genetics of GSSs, (ii) current approaches for precise engineering of analogous sexing systems, (iii) knowledge gaps and technical challenges in developing CRISPR-engineered GSSs in screwworm, and (iv) regulatory landscape governing genome-edited insects in the Americas in comparison to other contemporaneous biotechnologies. We argue that establishing a screwworm GSS would provide immediate benefits to existing management programs and hope this synthesis will inform and encourage future studies on this effort.

Keywords: Calliphoridae, blowflies, myiasis, CRISPR/Cas9, genome-editing

Introduction

Few insects have inspired as much popular fear and despise as the New World screwworm *Cochliomyia hominivorax* (Coquerel). Those feelings are not entirely irrational, and historical accounts from ranchers in the North American West recount grim cases of cattle being consumed alive by screwworm infestations (Schlitz et al. 2025). In contrast to closely related blowflies (family Calliphoridae), which are commonly found feeding and breeding on decaying organic matter (Stevens and Wallman 2006, Stevens et al. 2006, Yusseff-Vanegas and Agnarsson 2016), screwworm females rely on odors emitted from susceptible, living animals to find suitable oviposition sites (Thomas 1993, Spradbery 1994, Hall 1995). They deposit their eggs on dried margins of wounds and mucosal surfaces of their selected host, and within hours, hatching larvae burrow "head-down and closely packed" (Knipling 1960) into the host tissue, feeding on its living flesh (Thomas 1993, Hall and Wall 1995). Larval-infested wounds increase the production of semiochemicals that act as animal host-finding cues, attracting other nearby screwworms and facultative blowflies to lay their eggs on the same host (Chaudhury et al. 2010). These traumatic infestations often lead to severe debilitation and can result in death if untreated or untreatable. After feeding for about 5 days, the larvae crawl out from the host animal and burrow into the soil to pupate, later emerging as a new generation of flies. Mated females become ready to oviposit approximately

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5 days after emergence, laying discrete clutches of ~200 to 300 eggs at a time in a 3-day cycle over a lifespan of 2 to 3 weeks (Knipling 1960, Thomas 1993), potentially producing thousands of offspring in her lifetime. This form of dipteran (ecto) parasitism is termed primary, obligate wound myiasis, in which the fly larvae must feed on the host's living tissues to complete their development (Zumpt 1965, Hall and Wall 1995). Screwworms can infest almost all warm-blooded vertebrates, including humans—particularly those in elevated socioeconomic vulnerability (Hall et al. 2016, Moo-Llanes et al. 2026).

Given the serious implications of its life history traits, *C. hominivorax* is recognized as a high-consequence pest, causing severe suffering in domestic animals and wildlife, posing substantial threats to public health and food security, and generating significant economic losses for the livestock industry (Vargas-Terán et al. 2021, USDA-APHIS 2025). Accordingly, it is not surprising that the screwworm was elected the first target of the sterile insect technique (SIT) (Knipling 1955, Knipling 1960). *C. hominivorax* is native to the Americas, originally ranging from the southern United States and throughout Central and South America, with few exceptions (Vargas-Terán et al. 2021). However, by the early 2000s, the species had been effectively eradicated from continental North and Central America and several Caribbean islands through SIT. Despite this unprecedented achievement, screwworms remain a persistent concern in most regions of South America. Indeed, *C. hominivorax* has recently been identified as the leading candidate for deliberate global extinction (Kaebnick et al. 2025). The eradication of screwworms through a nearly 55-year area-wide integrated pest management (AW-IPM) campaign is widely regarded as a textbook example of the feasibility and effectiveness of the SIT. The coordinated effort resulted in the establishment of a continuously SIT-sustained biological barrier at the treacherous Darién Gap along the Panama–Colombia border (Klassen et al. 2021, Vargas-Terán et al. 2021), later managed by the COPEG (Panama-United States Commission for the Eradication and Prevention of Screwworm; https://www.copeg.org), which has excelled at keeping southern endemic and outbreak screwworm populations at bay for nearly 2 decades (Zhang 2020).

The last major resurgence of the screwworm in the United States caused a devastating 2016–2017 outbreak in the Florida Keys, resulting in the deaths of approximately 10% of the remaining endangered Key deer population before invasive flies were eliminated (Skoda et al. 2018). Between 2022 and 2023, Panama experienced a massive surge in screwworm cases, increasing from dozens of sporadic reports to thousands within a single year (Hailu et al. 2025, Keatts et al. 2025, Zhang 2025), leading to a nationwide outbreak. Screwworms slowly spread northward through Costa Rica, reaching Nicaragua a few months later. From there, they rapidly expanded across Central America, and less than a year later, Mexico reported its first case of screwworm-caused myiasis in the state of Chiapas, more than a thousand miles away. The first confirmed detection of screwworm in the United States occurred in June 2026, and as of this writing, active *C. hominivorax* cases remain confined to southwestern Texas (https://www.screwworm.gov; accessed 10 August 2026). Since its return, it is estimated that screwworms have infected thousands of animals and hundreds of humans across Central American countries and Mexico (https://www.cdc.gov/new-world-screwworm/situation-summary; accessed 10 August 2026).

Several interacting factors likely contributed to the screwworm's sudden resurgence, including suboptimal livestock management, increased—and often illegal—animal movement, and widespread deforestation in Mesoamerica (Keatts et al. 2025, Zhang 2025). Together with climate and ecological changes, these factors facilitate fly dispersal and create newly suitable habitats for establishment (Valdez-Espinoza et al. 2025, Moo-Llanes et al. 2026). Another largely unexplored possibility (but see Bush and Neck 1976, Bush et al. 1976, Richardson et al. 1982) is the genetic divergence of important phenotypic traits, such as mating behavior and chemical communication, driven by inbreeding and leading to partial sexual incompatibility between long-established production strains of *C. hominivorax* and wild populations, thereby reducing SIT efficacy.

Such a multifaceted scenario will require an equally coordinated and holistic response over the coming years to bring screwworm populations back under control (Akbari 2026, Andrews et al. 2026), echoing the remarkable international scientific and political collaboration that enabled its original eradication (Klassen et al. 2021). Sterile-fly dispersal within Panamanian territory has ceased, and a new "buffer zone" is being established along the United States–Mexico border in the state of Texas (Polansek and Douglas 2025, USDA 2026). If screwworms keep advancing, however, the containment area could expand severalfold, creating substantial logistical and economic challenges. Even if the invasion is successfully stanched at this stage, the cost of a subsequent multinational eradication effort to re-establish the sterile-fly barrier in Panama would represent a significant and sustained long-term burden on control programs.

It is widely recognized that a major operational limitation of standard SIT is the need to rear, sterilize, and release both sexes of the target species, despite only the male flies being effective for population suppression (Knipling 1960). Accordingly, it has also long been established that SIT is more effective when only sterile males are released, primarily by reducing mating competition from sterile females (Franz et al. 2021). The significantly enhanced cost-effectiveness and field efficacy of male-only releases were demonstrated in the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) (McInnis et al. 1994, Hendrichs et al. 1995, Cáceres et al. 2004, Rendón et al. 2004), decades after the original SIT campaign against *C. hominivorax* was launched, which relied—and continues to rely—on the co-release of sterile females (Scott et al. 2017). Notably, Edward Knipling, widely regarded as the father of SIT, originally referred to SIT as the "sterile-male method" (Knipling 1960), emphasizing that sterility is introduced into wild populations exclusively through sterile males. This biological insight guided fundamental research toward the development of strains enabling sex separation during early development (Whitten 1969, Robinson 2002). These so-called genetic sexing strains (GSSs) are considered one of the most revolutionary innovations in large-scale operational SIT within AW-IPM programs, forming a cornerstone of modern genetic-based biocontrol of invasive true fruit flies (family Tephritidae) of agricultural importance.

In a GSS, large-scale separation of males and females (sexing) is enabled by linking a dominant wild-type allele of a selectable marker (typically an autosomal recessive mutation) to the male-specific sex chromosome. This results in a system in which males and females differentially express the marker, thereby

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allowing sexing (Franz et al. 2021). Traditionally, GSSs are built by combining the isolation of suitable markers, with classical Mendelian genetics, and irradiation-induced chromosomal translocations. However, although several successful systems have been developed using classical genetics, most notably in medfly (Augustinos et al. 2017), those strains require substantial effort to be generated and maintained over time. Pure-breeding mutant lines and sexing strains produced by random mutagenesis frequently exhibit unintended effects on fitness, expressivity, or stability owing to the random nature of these methods, and breakdown due to recombination of translocated chromosomes. Additionally, traditional GSSs are inherently semi-sterile, as only half of the gametes from translocated "rescued" males are genetically balanced and viable (Franz et al. 2021), thereby reducing overall mass-rearing productivity. To address these limitations, it has been proposed that analogous GSS systems can be rationally engineered and (re)created in diverse pest insect species, with minor design modifications, by leveraging the CRISPR/Cas9 gene-editing toolbox (Yan et al. 2023, Davydova and Meccariello 2025, Davydova et al. 2026, Petrucci et al. 2026). We refer to such analogous systems as "CRISPR-engineered Genetic Sexing Strains."

Considering the expanding threat of the screwworm, as well as recent advances in essential genomics resources for this species—including high-quality genome assemblies (Scott et al. 2020, Tandonnet et al. 2023, Hickner et al. 2026), several transcriptomic data (nearly 100 Illumina RNA-seq entries on NCBI, totaling over 500 billion sequenced bases; accessed 25 April 2026), and established CRISPR/Cas9 protocols (Paulo et al. 2019, Paulo et al. 2022a)—we argue that now is a critical window of opportunity to modernize the strains supporting screwworm SIT programs. In the following sections, we build a case for the development of CRISPR-engineered GSSs in *C. hominivorax* by translating decades of GSS foundational research, development, and implementation in fruit flies. Other emerging biotechnologies are under development in the screwworm (eg Kaebnick et al. 2025, Akbari 2026), including increasingly popular conditional female lethal genetic systems (fsRIDL). However, we argue that the development of CRISPR-engineered GSSs based solely on endogenous genes may enable more rapid regulatory integration and near-term operational deployment within existing SIT infrastructure. In this context, modernization through GSS development represents an incremental improvement and a strategic step toward strengthening long-term screwworm control efforts, particularly as the scientific, regulatory, and social landscapes surrounding other contemporary biotechnologies continue to evolve.

The General Issue With SIT Bisexual Releases

The SIT is arguably the most successful example of an eco-friendly, autocidal pest control strategy in modern AW-IPM programs. Revolutionary ideas are often conceptually simple, and the operational principle of SIT illustrates this principle: mass-reared insects are sterilized by exposure to ionizing radiation and released in overwhelming numbers, so that sterile males outcompete wild males for access to wild females. Irradiated males transmit dominant-lethal mutations to their progeny, resulting in inviable embryos, thereby progressively suppressing and eventually eradicating the targeted natural population (Knipling 1960). In operational programs, the species of interest is first domesticated to artificial rearing conditions and maintained as laboratory-adapted wild-type lines capable of large-scale production. Sterilization is most commonly performed during the immobile pupal stage using relatively high dosages of X-ray or gamma radiation. Despite these exposures, the majority of treated pupae develop normally into sexually mature adults; however, their germline cells harbor extensive chromosomal damage. This damage does not typically impair adult mating, but induces genetic lethality in their offspring, as fertilized eggs derived from these gametes fail to complete embryogenesis (Robinson 2021).

A persistent operational limitation of conventional SIT is that both sexes are sterilized and released. In screwworm programs, this has been considered acceptable because sterile females do not exacerbate host injury; that is, they do not blood-feed or sting their hosts during oviposition, and sterile eggs laid on wounds do not develop into feeding larvae. Nevertheless, bisexual strategies carry 2 important costs. First, female *C. hominivorax* require higher irradiation doses than males to achieve complete sterility and eliminate residual egg hatching (Knipling 1960). As radiation doses increase, so does the likelihood of adverse effects on fitness, potentially compromising male survival and mating competitiveness (Robinson 2021). Because SIT success is fundamentally a competition between sterile and fertile males in nature, even modest reductions in fitness can significantly decrease its efficiency. Additionally, in the absence of co-released females, sterile males have greater odds of outcompeting wild males for wild females, increasing SIT efficacy (Hendrichs et al. 1995, Rendón et al. 2004). Second, bisexual releases effectively double production requirements, as half of the insects sterilized, transported, and released have little or no impact on the transmission of sterility into wild populations. In large-scale screwworm programs, where tens to hundreds of millions of flies may be released weekly, the need to manage sterile females represents a substantial financial and logistical burden (as discussed by Concha et al. 2016), even though they are biologically necessary for colony maintenance. To put into perspective, COPEG released ~15 million sterile screwworm flies per week to maintain the Darién Gap's barrier (Scott et al. 2017), with a maximum production capacity of ~100 million flies per week. Recently, the USDA estimated that at least 5 times this number would need to be released weekly to push *C. hominivorax* back to that same buffer zone (Polansek and Douglas 2025, USDA 2026).

Recognizing these limitations, Knipling suggested that "a more desirable way to utilize the sexual-sterility principle would be to induce sexual sterility [within] the natural population" itself (Knipling 1960), a concept that closely resonates with modern transgenic biotechnologies (eg Kaebnick et al. 2025, Akbari 2026). However, somewhat contemporaneously with the original screwworm eradication campaign in Mexico (Klassen et al. 2021), an alternative and more immediately practical solution to bisexual releases was being developed. Grounded in classical Mendelian genetics, Whitten (1969), working in the Australian sheep blowfly *Lucilia cuprina* (Wiedemann)—a close relative to the screwworm—demonstrated that geneticists could exploit recessive autosomal mutations producing scorable phenotypes early in development to enable sexing prior to sterilization and release. His approach relied on the linkage of these scorable genetic markers to the male-determining chromosome, so that the mutant phenotype

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would no longer segregate independently of sex. Systems built upon this principle became later known as GSSs.

Classical GSSs

Classical GSSs rely on 2 key components: a selectable genetic marker in the form of an autosomal recessive mutation producing a visual or conditionally lethal phenotype, and a dominant wild-type (rescue) allele of the same marker physically linked to the male-determining chromosome, ideally in close proximity to the maleness (M) factor (Franz et al. 2021). This genetic arrangement ensures that only males inherit a copy of the rescue allele. As a result, males are always heterozygous for the marker and display the normal, wild-type phenotype. Females, on the other hand, are homozygous recessive for the marker and display the mutant phenotype. Thus, this *de novo* sex-specific expressed trait enables sexing (Fig. 1).

The development of classical GSSs follows a well-established genetics framework (eg Zepeda-Cisneros et al. 2014, Ramírez-Santos et al. 2021). First, markers are identified and isolated through genetic crossings into pure-breeding lines. Suitable recessive mutations arise naturally in laboratory colonies or are induced through chemical or radiation-based mutagenesis. The selected marker must produce an unambiguously scorable phenotype, ideally early in development, with minimal fitness costs. A discrete population in which males carry the marker's dominant allele must also be established. Sex linkage is achieved by transferring the rescue gene into the male-determining chromosome, most commonly through irradiation-induced chromosomal translocations (Robinson 2002, Franz et al. 2021). In higher Diptera (suborder Brachycera), sex determination is generally governed by an M-factor located on the Y chromosome (Bedo and Foster 1985, Willhoeft and Franz 1996, Saccone et al. 2002, Scott et al. 2014), making it the natural target for linkage. Low-dose irradiation induces random chromosomal breaks, leading to reciprocal translocations between autosomes and the Y chromosome. If the translocated autosomal segment contains the wild-type allele, it becomes physically restricted to males, establishing the desired association between phenotype and sex.

Translocation-bearing males are crossed to homozygous mutant females, and stable sexing strains are obtained after several successive generations of selection. A central concern during this process is to achieve genetic stability. Meiotic recombination between the translocated segment and its homologous autosome counterpart can restore independent assortment of the marker, compromising the sexing system. This risk is reduced when the translocation breakpoint lies close to the M-factor (Franz et al. 2021). Genetic linkage studies in *C. hominivorax* revealed an apparent absence of recombination (crossing over) during meiosis in males (LaChance et al. 1966), a favorable biological characteristic for GSS development, as it reduces the likelihood of translocation breakdown.

While pioneered in blowflies (Whitten 1969, Robinson 2002), GSSs have become a cornerstone of SIT programs against invasive tephritid pests, with a prominent example being the VIENNA-8 strain of *C. capitata* (Augustinos et al. 2017, Franz et al. 2021; depicted in Fig. 1). Despite limitations in fitness and long-term stability (Franz et al. 2021, Petrucci et al. 2026), empirical studies on medfly GSS systems demonstrate that male-only releases substantially improve SIT performance relative to bisexual releases (see aforementioned references). Eliminating females from sterilization and release cohorts reduces the need for high radiation exposure, which can improve sterile males' mating performance, and increases the proportion of sterile males competing for wild females. Operational costs associated with irradiation, handling, transport, and release logistics also decrease. Decades of translational research on tephritid GSS systems provide a strong conceptual and technical foundation for transferring the

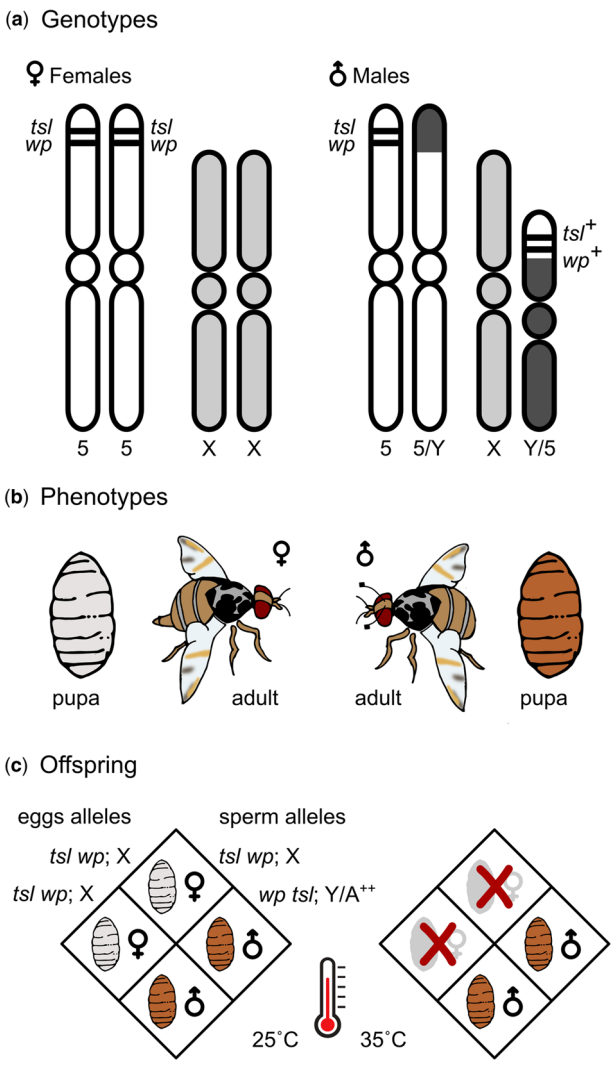

Fig. 1. Traditional structure of a genetic sexing strain. (a) Males carry a Y-autosome translocation linking the rescue alleles (+) of the genetic markers *white pupae* (*wp*) and *temperature-sensitive lethal* (*tsl*) to the male sex. (b) This ensures male-specific inheritance of "wild-type" phenotypes, including the typical brown puparium. On the other hand, females, homozygous for mutant alleles (*tsl* and *wp*), express the white puparium phenotype and are sensitive to elevated temperatures. (c) Under permissive conditions (25 °C), both sexes are recovered from offspring. However, under restrictive conditions (35 °C), the *tsl* mutation causes female-specific lethality, enabling male-only SIT releases. Only male haploid gametes generated through alternate segregation are shown, as these yield genetically balanced offspring; inheritance of the 5/Y autosome typically causes lethality during embryogenesis. X, X chromosome; 5, autosome 5; Y/5, translocation fragment carrying the Y centromere; 5/Y, reciprocal fragment carrying the autosomal centromere. Adapted from Franz et al. (2021).

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technology to the screwworm. Using these studies as a roadmap, the remaining challenge is to translate these principles into rationally engineered systems for *C. hominivorax*.

Genetic Markers

Ideal genetic markers used in automated large-scale screening share a few key characteristics. First, they are monogenic, recessive mutations producing reliably scorable phenotypes. Markers expressed at earlier developmental stages (eg embryo or larva) are preferable, as they would enable sexing before resource-intensive rearing. However, few of them have been well characterized in flies. An important exception is mutations causing conditional, temperature-sensitive lethal phenotypes in *Drosophila* and *C. capitata*. Consequently, pupal markers, although expressed relatively late in development, remain the best-supported candidates based on functional evidence. Second, rescue alleles should display clear dominant effects, with full penetrance and consistent expressivity, ensuring that males express the wild-type phenotype. Finally, they should have minimal effects on overall fitness, especially on reproductive performance and competitiveness of heterozygous rescued males.

Early genetic studies of *C. hominivorax* identified multiple mutant phenotypes (Lachance and Hopkins 1962, LaChance et al. 1966, Taylor and Cuevas 1986, Taylor 1989). Interestingly, none of them appear to correspond directly to those successfully used in GSSs of tephritids. In fruit flies, mutations affecting pupal color and temperature-dependent lethality have proven particularly useful for developing GSSs. In recent years, integrative genetics and genomics have clarified the molecular basis of major markers (Ward et al. 2021, Aumann et al. 2025, Paulo et al. 2025), collectively providing a blueprint for reverse genetics in the screwworm. Here, we discuss the suitability of these mutations, providing a basis for establishing gene orthology and functional conservation in the screwworm (Table 1; Supplementary Data S1).

White Pupae

The white pupae or white puparium (wp) is a key phenotype of GSS development in major horticultural tephritid pests (Robinson and Van Heemert 1982, McCombs and Saul 1995, McInnis et al. 2004) and is used in medfly SIT programs worldwide (Augustinos et al. 2017). As in screwworm, medflies develop an opaque brownish or dark reddish-brown puparium. In contrast, flies carrying the *wp* mutation develop a conspicuous opaque, enamel-white puparium, with adults having a wild-type appearance. Genetic studies on medfly established *wp* as a single autosomal recessive locus on chromosome 5 (Rossler 1979, Rossler and Rosenthal 1992), later confirmed by cytogenetics (Kerremans and Franz 1994) and genomics (Sim et al. 2017). Biochemical evidence suggested that the mutation disrupts the transport of hemolymph catecholamines to the puparial cuticle, preventing normal sclerotization and pigmentation (Wappner et al. 1995). It was only nearly 40 years after its first description in the medfly that the genetic basis of the *wp* mutation was resolved. Ward et al. (2021) demonstrated that the wp phenotype in different fruit flies results from parallel mutations in a single conserved gene encoding a major facilitator superfamily (MFS) transporter. Functional validation *via* CRISPR/Cas9-mediated gene knock-outs (KOs) confirmed that loss-of-function mutations in *wp* loci are sufficient to produce the mutant phenotype across distantly related tephritids (Ward et al. 2021, Paulo et al. 2022b), suggesting an evolutionarily conserved function. Orthologs have not been validated outside Tephritidae, but a phenotypically similar recessive

Table 1. Summary of screwworm ortholog candidates for genetic sexing strain (GSS) development

Locus[a]	Marker	Identity[b]	Signatures	Phenotype	Mechanism	Evidence[c]	Suitability
CM118043.1: 2416302–2418137 (+)	*white pupae* (*wp*)	80.4%	16–458: MFS (IPR020846)	Puparium opaque enamel-white	Transport (likely transmembrane) of hemolymph pigment precursors to the puparial cuticle	Strong	High
CM118040.1: 103650040–103653092 (-)	*ebony* (*e*)	74.9%	26–410: AMP-binding (IPR000873) 35–549: A_NRPS (cd05930) 586–646: PP-binding (IPR009081)	Puparium shiny black; darkening of posterior larval spiracles; ectopic melanization of adult cuticle; may present fitness costs in homozygosity	Encodes NBAD-synthase that converts DA into NBAD; the precursor of light tan pigments in insect cuticles	Strong	Moderate
CM118043.1: 50482947–50485409 (+)	*temperature-sensitive lethal* (*tsl*)	83.9%[d]	147–256: LysRS_N (IPR044136) 259–597: LysRS_core (IPR018149)	Lethality at any developmental stage at temperatures near 35 °C; full embryonic lethality after 24 h heat-shock treatment at 34 °C	Unknown; likely involved in protein synthesis (charging tRNA–Lys); misfolding of the *tsl*-encoded protein at non-permissive temperatures may disrupt translation	Limited to medfly	Low

[a]Based on the most recent *C. hominivorax* reference genome assembly (GenBank: GCA_051144965.1). Additional information and manually curated gene models can be found in Supplementary Data S1.
[b]Calculated at the peptide level against *C. capitata* orthologues.
[c]Likelihood of conserved molecular function in screwworm, leading to analogous mutant phenotypes, based on historical accounts and functional validation in diverse species.
[d]Between screwworm *lysRS* variant 2 and medfly's isoform X2 (XP_004536423.1); signature motifs correspond to the same screwworm variant.

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mutation, *pale puparium* (*pp*), has been described in *L. cuprina*. The *pp* marker is located on the genetic mapping chromosome 2 and produces a "straw-colored puparium" (Foster et al. 1981), reminiscent of the wp phenotype, perhaps suggesting a conserved pupal pigmentation pathway between these flies.

Black Pupae

Naturally occurring wp phenotypes appear to be rare among schizophoran flies, which may reflect species-specific genetic constraints. For example, disruption of *wp* may be deleterious in some taxa—the *pp* mutants in *L. cuprina*, for instance, exhibit reduced size and viability (Foster et al. 1981)—or the phenotype may have a complex genetic basis. Consequently, many GSSs instead rely on the ubiquitous black pupae or black puparium (bp) phenotype (Zepeda-Cisneros et al. 2014, Meza et al. 2020, Ramírez-Santos et al. 2021, Cárdenas-Enríquez et al. 2026). In medfly, the historical *black pupae* and *niger* mutants produce a shiny black puparium, and often display darkening of the posterior larval spiracles and cuticle in adults. Biochemical analyses showed that both accumulate dopamine owing to defects in N-β-alanyldopamine synthesis; however, rescue assays demonstrated that the underlying mutations affect different steps of the yellow–tan branch of the insect melanin biosynthesis pathway (Wappner et al. 1996a, 1996b). These findings closely parallel the *black* (also known as *Adc*) and *ebony* mutants of *Drosophila melanogaster* (Meigen), suggesting homologous genes (Wappner et al. 1991). Consistent with this hypothesis, Paulo et al. (2025) identified *ebony* as the causal gene underlying the bp phenotype in the Guatemala 10 (GUA10) GSS of the Mexican fruit fly *Anastrepha ludens* (Loew). Cas9-mediated KOs produced analogous mutant phenotypes across distantly related tephritids, demonstrating strong functional conservation of *ebony*. Comparable melanic mutations have also been described in calyptrate flies, including the housefly *Musca domestica* (Hiroyoshi 1977) and the blowfly *L. cuprina* (Hackman and Goldberg 1968). Indeed, a *bp* mutation in *Lucilia* was instrumental in the development of the earliest GSSs in higher flies (Whitten 1969). Classical genetic studies in *C. hominivorax* described a marker termed *black* (*bl*), in which the "normal blue–green body coloration is changed to jet black" (Lachance and Hopkins 1962, LaChance et al. 1966). However, *bl* appears to be X-linked, affecting only adult pigmentation, making it unlikely to correspond to either *ebony* or *black*.

Temperature Sensitive Lethal

The isolation of the *temperature-sensitive lethal* (*tsl*) marker was pivotal in SIT programs against the medfly (Franz and McInnis 1995), and the development of the benchmark *tsl*-based GSS VIENNA-8 remains unique to the species (Augustinos et al. 2017, Franz et al. 2021). Homozygous *tsl*-female embryos die after 24 h at 34 °C, whereas rescued males survive, enabling selective elimination of females through simple water-bath immersion. As sexing can be achieved earlier in development, GSSs built around *tsl* are more efficient and cost-effective than systems based on pupal color mutations. Nevertheless, because *tsl* is not visually identifiable, a linked *wp* mutation is usually used to monitor treatment efficacy and recombination events (as shown in Fig. 1). In medfly, this is facilitated by the close proximity of the 2 markers on chromosome 5 (Sollazzo et al. 2023), allowing them to be translocated together onto the Y chromosome. The "*tsl* genomic region" has been closely investigated, and several *tsl* candidate genes identified, most notably *deep orange* (*dor*), which can trigger heat-sensitive phenotypes, albeit at higher temperatures than *tsl* (Sollazzo et al. 2024). The *tsl* causative mutation in *C. capitata* was identified as a point mutation leading to a histidine-to-tyrosine amino acid substitution in the *lysine-tRNA ligase* (*LysRS*) gene (Aumann et al. 2025). Its recreation *via* CRISPR/Cas9-mediated gene-editing reproduced the *tsl* phenotype, causing complete embryonic lethality under standard heat-treatment conditions. Conversely, *piggyBac*-mediated genome integration of a *mini-LysRS* gene restored the wild-type phenotype. An important question is whether the same mutation in the screwworm *LysRS* orthologue would produce a similar and stable tsl phenotype. Screwworm embryos are incubated at temperatures near 37 to 39 °C to mimic the warmth of a living animal host. These temperatures exceed the *tsl* embryonic threshold in medfly; thus, the mutation could be lethal in screwworms. If true, 1 possible solution would be to establish lines adapted to lower temperatures. Eggs of the historical GO05 strain of *C. hominivorax*, domesticated at the Center for Molecular Biology and Genetic Engineering (CBMEG) at the State University of Campinas (UNICAMP) in Brazil, were often reared at 29 °C with no obvious impact on hatching and larval development (D.F. Paulo, personal notes). Alternatively, mutations in other genes producing similar heat-sensitive phenotypes could be explored (Nguyen et al. 2021). An interesting candidate is the point mutation *E839K* in the *dor* gene (Sollazzo et al. 2024). Medfly embryos homozygous for this mutation display a 90% to 94% hatching rate at 34 to 35 °C, requiring temperatures above 36 °C for complete lethality, which is potentially better suited to screwworm rearing conditions.

Neoclassical GSSs

The discovery of the long-sought *wp* (Ward et al. 2021) and *Maleness-on-the-Y* (*MoY*) genes (Meccariello et al. 2019) in tephritids opened a new avenue for the rapid and reliable development of GSSs using CRISPR/Cas9 gene-editing methods (Doudna and Charpentier 2014). The first molecular strategy to be formally proposed was termed the "neoclassical" approach (Yan et al. 2023), where the classical genetic architecture of GSSs is recreated through genome engineering. Importantly, genome edits could be performed without introducing any foreign DNA, making the resulting strain amenable to diverse regulatory frameworks (discussed later). Of note, although the neoclassical approach is simple in theory, a proof of concept has yet to be presented. An in-depth review is given by Petrucci et al. (2026), and additional views can be found in Davydova and Meccariello (2025).

Assuming that orthologs for genetic markers exist and are functionally conserved, the establishment of a mutant line can be achieved through *in vivo* delivery of pre-assembled ribonucleoprotein complexes (RNPs) consisting of Cas9 protein and single guide RNA (sgRNA) targeting the intended loci. The resulting DNA double-strand breaks (DSBs) are primarily repaired by the error-prone non-homologous end-joining (NHEJ) pathway, often resulting in a variety of insertions and deletions (indels) events. When indels are induced in coding sequences, they usually lead to the introduction of premature stop codons. If these frameshift mutations are produced early on the gene sequence or at important protein functional

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domains, they increase the likelihood of effectively producing null mutants. If precise edits are desirable—for instance, to recreate the specific amino acid substitution of the *tsl*-related *LysRS* mutant allele (Aumann et al. 2025)—RNPs cocktail can be supplemented with a single-stranded oligonucleotide (ssODN) donor template (Aumann et al. 2018), preferably containing asymmetrical short homologous arms (Richardson et al. 2016). The donor template is used to guide precise DNA changes by exploiting the cell's homology-directed repair (HDR) pathway, thereby producing targeted gene edits.

As in traditional GSSs, neoclassical sexing systems would still require the linkage of rescue alleles to the male sex. In contrast to routine Cas9-mediated gene KOs, this requirement might be methodologically more challenging, and 2 alternatives have been proposed under the neoclassical approach:

Chromosomal Translocations

Sex linkage could be achieved by reciprocal translocation induced through the dual targeting of nonhomologous chromosomes (Chen et al. 2015); that is, by simultaneously inducing Cas9-mediated DSBs at specific positions on the Y and the autosome carrying the marker. These "dual-breaks" are preferentially repaired by the NHEJ and microhomology-mediated end-joining (MMEJ) pathways, leading to chromosome rearrangements (Brunet and Jasin 2018). CRISPR/Cas9-mediated chromosomal translocations have been achieved in *Drosophila* (O.S. Akbari, *unpublished*), but the approach still needs validation in other flies. Autosomal translocation-bearing strains of *D. melanogaster* have also been successfully generated using the rare-cutting nuclease I-SceI (Buchman et al. 2018), but a precise Y-to-autosome translocation may require the *de novo* creation of target sites beforehand. Alternatively, radiation-induced reciprocal translocations could be used to establish the desired linkage, following the crossing scheme of the classical genetic approach. Even though this method lacks the precision of targeted gene-editing, it may nonetheless represent a practical alternative. In the screwworm, this is especially attractive given the extensive knowledge on radiation-induced mutagenesis in the species.

Sex-specific Targeting

In addition to short repair templates, the HDR pathway can be used to insert larger DNA fragments into specific regions of the genome using double-stranded DNA (dsDNA) donor templates (eg Kistler et al. 2015). These donors can carry a "cargo" harboring the rescue allele of the marker, designed for direct insertion into a suitable Y chromosome "landing" site *via* CRISPR/Cas9-mediated gene knock-ins (KIs), as achieved in *Drosophila* (Buchman and Akbari 2019, Gamez et al. 2021). Sex-specific targeting offers several advantages over chromosomal translocations. First, the rescue gene can be directly inserted into the Y of a previously generated homozygous mutant line, eliminating the need to establish a separate translocated population. Second, because only the rescue allele is inserted into the male chromosome—rather than large autosomal segments—all the resulting male gametes are expected to be genetically balanced, eliminating the semi-sterility issues of classical GSSs (Franz et al. 2021).

It is important to notice, however, that efforts to insert complex gene sequences into the Y chromosome are hindered by its heterochromatic (ie highly condensed and generally transcriptionally inactive) and repetitive nature. As a result, multiple landing sites must be empirically tested to determine whether neighboring *cis*-regulatory DNA elements and chromatin structure influence expression of the rescue allele (Wimmer 2005). For example, Buchman and Akbari (2019) evaluated 10 candidate Y-linked insertion sites in *Drosophila*, yet only 2 supported successful HDR-mediated KI of fluorescently marked transgenes. Furthermore, KIs should ideally be positioned as close as possible to the M-factor to mitigate meiotic recombination. However, the identity of this locus remains unknown in most SIT-relevant species (but see Hopkins and Kopp 2021), including the screwworm. Despite major advances in genome sequencing and assembly for *C. hominivorax*, the notoriously difficult to reconstruct Y chromosome remains poorly characterized; only ~650 kb has been recovered (Hickner et al. 2026), likely representing a small fraction of its total length. Taken together, these constraints suggest that Y-linked KI strategies are technically challenging, albeit not impossible.

An interesting alternative strategy to dsDNA KIs would be to adapt the CRISPR-assisted insertion tagging (CRISPaint) method to achieve homology-independent insertion of rescue alleles (eg Bosch et al. 2020). Briefly, the method uses CRISPR/Cas9 to insert large DNA sequences at specific genomic locations by simultaneously cleaving the genome and a donor vector *in vivo*, allowing their integration through the cell's NHEJ and MMEJ pathways. Compared to HDR-based approaches, this method could facilitate the delivery and integration of larger constructs and simplifies donor template preparation (Bui and Kamiyama 2023).

It is generally accepted that the efficiency of HDR-mediated KIs decreases with increasing cargo size. Therefore, integrations into the Y would certainly benefit from minimal, intronless rescue gene constructs (mini-genes), which have recently been developed for the *wp* (Fan et al. 2025, Prates et al. 2026) and *tsl* (Aumann et al. 2025). Additionally, integrating sequencing technologies for profiling tissue-specific transcriptome (RNA-seq) and epigenome (ATAC-seq and ChIP-seq) could help identify *cis*-regulatory regions controlling the spatiotemporal expression of genetic markers, thereby further improving the design of rescue mini-genes.

Cisgenic Genetic Sexing Strains

New molecular strategies to (re)create GSSs are still emerging as the field evolves. Remarkably, Davydova et al. (2026) proposed a new method leading to the development of cisgenic genetic sexing strains (CGSSs), representing the first application of CRISPR/Cas9 to engineer a fully cisgenic sexing system (ie one that relies exclusively on the species' own DNA sequences). Their method involves the seamless HDR-mediated insertion of a known sex-specifically spliced intron into a selectable genetic marker. Because this strategy leverages conserved molecular pathways, it is conceptually generalizable and may be adaptable to a wide range of insect species.

The underlying core mechanism relies on sex-specific alternative splicing as a regulatory "switch" for sex-determination, which has been well characterized in many higher flies, including blowflies (Scott et al. 2014). The *transformer* (*tra*) gene represents one of the best-studied examples and has been widely used due to its well-characterized genetic structure and expression patterns. To illustrate: in *C. hominivorax*, *tra* transcripts are sex-specifically spliced such that only females

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produce a functional TRA protein required for their normal development (Fig. 2a). Meanwhile, males fail to excise the first *tra* intron, which harbors a "poison" exon (named M1) containing multiple in-frame stop codons, resulting in a truncated, non-functional protein (Li et al. 2013). The default state of the system is programmed for female development, triggered by maternally deposited female-specific *tra* mRNA in embryos, which initiates the production of TRA protein through a positive feedback loop. In males, this system is turned "off" by the M-factor, which likely resides on the Y chromosome. Similar to RNA interference (RNAi)-mediated knockdown in other blowflies (Concha and Scott 2009, Li et al. 2013), CRISPR/Cas9-mediated disruption of *tra* in *C. hominivorax* results in the masculinization of females (Paulo et al. 2019), providing functional evidence for the conservation of this system in screwworm. It is noteworthy that this natural, early-acting sex determination pathway has been successfully exploited to create transgenic sexing strains in screwworm (Concha et al. 2016, Concha et al. 2020), demonstrating that it can be harnessed to control the expression of other genes in a sex-specific manner.

Building on this principle, Davydova et al. (2026) seamlessly inserted the first intron of *tra* into the *wp* gene in *C. capitata* genome, creating a novel sexing system in which correct splicing and functional WP protein production occurs only in females (Fig. 2b). Using this elegant minimal cisgenic modification (a ~1.3 kb *tra* intron insertion into the *wp* locus, both from the same species), the authors generated a homozygous sexing strain in the medfly, named IMPERIAL. In this strain, females display the wild-type brown pupal phenotype, whereas males exhibit the mutant white puparium phenotype, enabling straightforward visual or automated sexing (Fig. 2b). Despite effectively inverting the classical GSS configuration (as shown in Fig. 1b), the IMPERIAL CGSS demonstrated improved genetic stability relative to the benchmark VIENNA-8 and maintained fitness levels comparable to its wild-type background, indicating no obvious pleiotropic effects of the modification.

A key advantage of this approach is that it eliminates reliance on exogenous regulatory elements, thereby reducing risks of position effects commonly associated with *piggyBac*-mediated integration and likely to affect inserted genes into the Y. Since cisgenic GSSs involve a small, precise modification within a single autosomal exon, they are also expected to be less susceptible to recombination-mediated breakdown, offering greater long-term genetic stability than other GSS designs discussed here. Lastly, because they are built entirely from endogenous sequences, CGSSs may better align with multinational biotechnology regulatory frameworks.

Some biological and practical considerations remain before the cisgenic approach can be generalized across species. For instance, it is unclear whether similar KIs into pleiotropic markers, such as *ebony* (discussed by Paulo et al. 2025), produce viable strains. In addition to *tra*, intronic elements from the downstream sex-determination genes *doublesex* (*dsx*) and *fruitless* (*fru*) (Hopkins and Kopp 2021) may provide additional candidates for engineering CGSSs, further expanding the approach's toolkit. Nonetheless, the cisgenic strategy offers a simpler and robust alternative to both classical and neoclassical approaches. If successfully implemented in *C. hominivorax*, it could represent a highly scalable and regulatory-compatible platform for next-generation SIT strains.

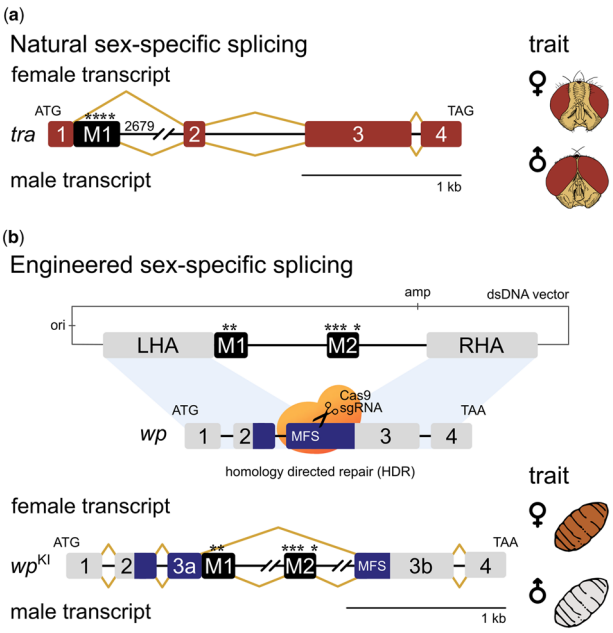

Fig. 2. Leveraging sex-specific splicing mechanisms to build cisgenic GSSs. (a) In *C. hominivorax*, maternally deposited transformer protein (TRA) initiates an autoregulatory loop—later maintained by zygotic TRA—resulting in correct splicing of *tra* mRNA and production of a functional protein. In males, expression of an M-factor on the Y chromosome interrupts this loop, leading to alternative splicing that retains the first *tra* intron. This region contains a "poison" exon (M1) with multiple in-frame stop codons (*), resulting in a truncated, nonfunctional protein. These sex-specific TRA variants direct alternative splicing of downstream genes, finally determining sex-specific traits (eg holoptic males and dichoptic females). (b) The cisgenic approach leverages this natural sex-specific alternative splicing mechanisms to achieve sex-specific marker expression. A proof-of-concept was developed in *C. capitata*, where the first intron of the *tra* gene (1,345 bp) was seamlessly inserted into the *wp* locus using CRISPR/Cas9 HDR-mediated knock-in (KI). In the resulting strain, this novel engineered sex-specific splicing allele (wp^{KI}) confers sex-specific expression of the white puparium trait, enabling early sexing. Exons are shown as numbered boxes; introns as black lines; and splice junctions as yellow lines. M1 and M2, male-specific exons; LHA and RHA, left and right homology arms (~700 bp); MFS, major facilitator superfamily domain. Adapted from Pane et al. (2002), Li et al. (2013), and Davydova et al. (2026).

Transferring the GSS Technology to the Screwworm

Despite advances in genomics and transgenics, comparatively little progress has been made in expanding the CRISPR/Cas9 toolbox for the screwworm. Encouragingly, the broad literature on targeted gene-editing in insects provides the methodological foundation for implementing similar approaches in *C. hominivorax*. Here, we outline key technical challenges and highlight promising strategies to overcome them, with the goal of facilitating the development of GSSs in the screwworm.

Cas9-mediated KOs

Despite sequence conservation, candidate markers must be functionally validated. KO experiments should confirm that these genes share similar molecular functions and phenotypes in screwworm to those previously discussed. Once this is

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determined, pure-breeding mutant lines can be established and evaluated for their potential use in GSS development. Protocols for CRISPR/Cas9-mediated KOs have been established for *C. hominivorax* (Paulo et al. 2019, Paulo et al. 2022a) based on methods developed for *D. melanogaster* (Bassett et al. 2013, Bassett and Liu 2014) and *C. capitata* (Meccariello et al. 2017, Aumann et al. 2018). Briefly, pre-assembled Cas9–sgRNA RNPs are microinjected into the posterior end of early, non-dechorionated preblastoderm embryos (less than 60 min after egg laying). Injection cocktails consist of purified Cas9 protein (360 to 500 ng/µl) and *in vitro*-transcribed sgRNAs (200 ng/µl) prepared in sodium phosphate buffer supplemented with a high concentration of KCl (300 mM), which improves RNP solubility (Burger et al. 2016). Higher Cas9 concentrations maximize mutagenesis, while lower concentrations provide a better balance between editing efficiency and embryo survival, facilitating the recovery of independent homozygous mutant lines. Accordingly, a final Cas9 concentration of 360 ng/µl has been proposed as a general standard for fruit flies (Paulo et al. 2022b) and has proven sufficient to generate heritable biallelic mutations in *C. hominivorax* (Paulo et al. 2019).

Cas9-mediated HDR

Efficient CRISPR/Cas9-mediated HDR protocols are essential for developing CRISPR-engineered GSSs. Successful HDR-mediated KIs have been achieved in *L. cuprina* (Williamson et al. 2021, Wulff et al. 2025). In these studies, dsDNA donors were assembled using modular cloning approaches (Golden Gate or Gibson) and the cassette consisted of a fluorescent reporter gene driven by a constitutive promoter, flanked by a pair of ~0.7 to 1 kb homology arms (Concha et al. 2011) corresponding to sequences immediately upstream and downstream of the sgRNA target site (similar to Fig. 2b). Repair templates were co-injected with pre-assembled RNPs at a final concentration of 500 ng/µl. Offspring from backcrossed flies were screened for fluorescent marker expression, and successful KIs were recovered at frequencies of approximately 10%. Although repair templates concentration used in *Lucilia* is consistent with early studies (Gratz et al. 2014, Kistler et al. 2015), more recent protocols in fruit flies typically use lower concentrations (200 to 300 ng/µl) (Aumann et al. 2018, Aumann et al. 2020, Meccariello et al. 2024, Davydova et al. 2026), which may improve embryo survival and facilitate microinjections. While HDR-based KI remains a key technical bottleneck in screwworm, its successful implementation in related species suggests that this challenge is primarily one of optimization rather than feasibility.

Increasing KI Efficiency

Cas9-mediated KI efficiency is known to be substantially lower than that of KOs (see Zirin et al. 2022 and references therein), in part because the underlying DSB repair pathways differ in their activity during the cell cycle; while NHEJ operates throughout the cell cycle, HDR is largely restricted to the S and G2 phases, when sister chromatids serve as homologous templates. Consequently, strategies that bias repair toward HDR may improve KI efficiency. For example, transient inhibition of key NHEJ components, such as DNA ligase IV (*Lig4*) or the Ku70/80 complex, favors repair through homology-directed mechanisms (Beumer et al. 2013, Basu et al. 2015, Zhu et al. 2015). This could be achieved by co-injecting short interfering RNAs (siRNAs) targeting their corresponding mRNAs or by generating intermediate NHEJ-deficient KO lines. In *Drosophila*, HDR frequencies also increase when CRISPR components and repair templates are co-injected into transgenic Cas9-expressing lines (Gratz et al. 2014, Gratz et al. 2015). Germline-specific promoters from *nanos* and *vasa* genes are commonly used to drive Cas9 expression (eg Meccariello et al. 2024), and orthologues have been identified in the *C. hominivorax* genome (Scott et al. 2020). In cisgenic GSS designs, the repair cassette should also include a fluorescent marker driven by a constitutive promoter (such as *Ubi*, *hsp70*, or *hsp83*, commonly used in screwworm studies) to facilitate subsequent "clean-up" of the transgene from the resulting KI line. This can be accomplished through genetic crosses and selection of offspring that have lost the fluorescent marker, thereby restoring a non-transgenic state. Because transgenes would be removed, intermediate Cas9-expressing lines could be generated using well-established *piggyBac* transposon-based transformation protocols available for screwworm (Concha et al. 2016, Concha et al. 2020).

Biotechnologies and Regulatory Frameworks

Modern biotechnology advances have enabled the design and development of several genetically engineered transgenic systems for screwworm management and direct field population suppression (see review by Akbari 2026). Here, we briefly outline them in the context of regulatory comparisons with the classical and CRISPR-engineered GSSs (Table 2), as regulatory considerations are a central determinant of whether biotechnologies move from laboratory to field deployment.

The most advanced sexing system developed in *C. hominivorax* is the female-specific release of insects carrying dominant lethal (fsRIDL) (Concha et al. 2016, Concha et al. 2020, Lowman et al. 2025, Arp et al. 2026). These systems enable both pre-release sexing and local population suppression through the inheritance of a conditional female-specific lethal trait. Similarly, CRISPR/Cas9-based homing gene drive (HGD) (Esvelt et al. 2014, Gantz and Bier 2015, Champer et al. 2016) are designed to suppress populations by biasing sex ratios toward males through self-propagation across generations, an approach currently under investigation for *C. hominivorax* (Fresia et al. 2021). A combination of both strategies has even been proposed as "sufficiently promising" to achieve complete screwworm eradication (ie deliberate extinction) (Kaebnick et al. 2025). Another transgenic approach for sexing is the SEPARATOR system, which uses engineered sex-specific alternative splicing of fluorescent markers to enable automated sorting during early development (Davydova et al. 2023, Weng et al. 2023). Finally, an emerging CRISPR-based technology termed precision-guided SIT (pgSIT) (Kandul et al. 2019) crosses Cas9- and sgRNA-expressing parental lines to simultaneously produce sterile, male-only progeny, offering a self-limiting approach to population suppression. To date, neither SEPARATOR nor pgSIT has been investigated in screwworm, but a case for these technologies has been presented by Akbari (2026).

A key consideration for this forum is that all of these systems rely on the introduction of foreign DNA into the species' genome; a transgenic condition. This distinction largely determines how technologies are classified, regulated, and ultimately deployed. While undeniably powerful, transgenic systems are

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Table 2. Comparison between promising genetic sexing systems for the screwworm generated by different biotechnologies

Approach	Main mechanism	Core modification	Main advantages	Main limitations	Regulation	Feasibility
Classical GSS	WT phenotype rescue *via* Y-linkage	Irradiation-induced Y-autosome translocation	Proven	Random mutagenesis; recombination-prone; labor-intensive; requires sterilization	Highly favorable (non-GMO)	High
Neoclassical GSS[a]	WT phenotype rescue *via* Y-linkage	Cas9-mediated KO genetic markers + HDR-mediated KI of rescue genes into Y	Rational design; no foreign DNA; genetically stable (mediate landing site on the Y)	Suitable marker identification; efficient HDR needed; requires rescue gene constructs; Y targeting difficult; relatively late detection of marker (pupae); requires sterilization	Favorable (likely non-GMO)	Moderate
Cisgenic GSS	Sex-specific alternative splicing of genetic marker	Targeted HDR-mediated KI of sex-specific intronic region	Proof-of-concept in medfly; no foreign DNA; genetically stable	Suitable marker identification; efficient HDR needed; relatively late detection of marker (pupae); requires sterilization	Favorable (likely non-GMO)	High
SEPARATOR	Sex-specific splicing of fluorescent marker	*piggyBac*-mediated transgene integration	Early detection of marker (larvae); large scale, highly accurate automated sexing	Transgenic; fitness costs due to position effects; regulatory barriers; requires sterilization	Restrictive	High
fsRIDL[b]	Dominant female conditional lethality	*piggyBac*-mediated transgene integration	Established in screwworm; highly effective	Transgenic; unintentional tetracycline impacts on strains; regulatory barriers; released males are fertile[c] (if not coupled with SIT)	Restrictive	High
pgSIT	CRISPR/ Cas9-mediated disruption of female viability and male fertility in progeny	*piggyBac*-mediated integration of transgenes expressing Cas9 and sgRNAs	Simultaneous sexing and sterilization; self-limiting; no persistence/spread	Requires maintenance and crossing of 2 transgenic parental lines (Cas9 and sgRNAs)[d]; produce intersex females	Restrictive	Moderate
HGD	Biased inheritance of drive disrupting female viability	HDR-mediated KI of drive construct	Fewer releases; Potential long-term suppression	Transgenic; released males are fertile[c]; "resistance alleles" could arise; potentially uncontrollable persistence/spread; regulatory and ethical barriers	Highly restrictive	Low

[a]A comprehensive comparison between classical and neoclassical GSSs is given by Petrucci et al. (2026). For this comparison, we are assuming linkage of functional rescue genes *via* the "sex-specific targeting" method (discussed in the main text).
[b]A comprehensive description of fsRIDL systems in the screwworm will be given in Arp et al. (unpublished).
[c]Although female progeny is eliminated, surviving male offspring (maggots) develop normally and can contribute to myiasis until population suppression is achieved.
[d]A newer version of the system presented in Kandul et al. (2021) eliminates the requirement of 2 independent lines by conditionally driving the expression of Cas9 with the heat-chock inducible promoter *Hsp70Bb*.

often subject to stricter regulatory oversight and lower public acceptance, particularly self-propagating systems such as gene drives, which frequently raise environmental ethics debates (see Boersma et al. 2023 and references therein). These factors have historically hindered large-scale testing and deployment. Importantly, regulatory oversight is driven primarily by the presence of foreign DNA rather than by the genome-editing process itself.

In contrast, CRISPR-engineered GSSs can be designed to avoid transgenic elements entirely by modifying only endogenous sequences of the target species, a cisgenic condition. As such, they may fall outside traditional GMO (genetically modified organism) classifications and regulations. Genome-edited (GnEd) organisms are commonly classified into 3 categories based on the resulting genetic modification (Wray-Cahen et al. 2024). SDN-1 comprises small Cas9-induced indels generated without donor templates, typically producing gene KOs. Template-guided edits are further divided into SDN-2, which includes small sequence changes introduced using ssODNs, and SDN-3, which involves the insertion of novel genetic material into the original genome using dsDNA donors. This distinction is particularly relevant for modern screwworm eradication campaigns, as SIT programs operate across multiple countries with different regulatory frameworks and may therefore influence how CRISPR-engineered GSSs are integrated into them.

Regulatory frameworks are dynamic and vary considerably among countries. Although the literature on GnEd-derived insects remains limited, CRISPR-engineered GSSs are likely to be considered non-GMOs in several South and Central

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American countries, provided they are free from foreign DNA. Such strains are generally regarded as equivalent to those obtained through conventional breeding or traditional mutagenesis (Sprink and Wilhelm 2024, Wray-Cahen et al. 2024) and may therefore be evaluated more flexibly, with reduced regulatory complexity (eg Hoffman 2021). By contrast, Mexico's stance toward GnEd systems remains unclear and, until such distinctions are established, they are likely to be treated as traditional GMOs (Kuiken and Kuzma 2021). In the United States, there is no specific legislation for bioengineered organisms, and "products" are evaluated on a case-by-case basis according to intended use, environmental impact, and potential for persistence or spread (Wray-Cahen et al. 2024). Self-sustaining biotechnologies such as gene drives introduce additional regulatory and ethical complexity, given their potential for uncontrolled spread and long-term ecological impacts (Esvelt et al. 2014, Oye et al. 2014).

Unlike them, CRISPR-engineered GSSs are intended exclusively for use within SIT programs. As these insects are sterilized prior to release, engineered alleles are not expected to persist in natural populations. SIT has a decades-long history of environmentally safe, species-specific use against agricultural pests, most notably screwworm and medfly, with strong community support. The original screwworm eradication campaign and the long-standing SIT barrier at the Panama–Colombia border are both testaments to this (Zhang 2020). Similarly, the US state of California was only able to stop the so-called "medfly wars" during the 1980s with the deployment of SIT (Haberman 2014, Arredondo 2022). Since the 1990s, the Preventive Release Program has successfully relied on SIT to keep persistent medfly populations at bay (Barry et al. 2004, Hendrichs et al. 2021) and has long benefited from the development of operational GSSs (Franz and McInnis 1995). These precedents suggest that CRISPR-engineered GSSs may face fewer ethical and regulatory barriers than transgenic-based population suppression technologies, making them an attractive near-term option for screwworm management.

Closing Remarks

There is broad agreement that current screwworm SIT programs would benefit substantially from the development of sexing systems. The key question is not whether such systems are needed, but which biotechnology offers the most practical, timely, and ethical path forward. We argue that CRISPR-engineered GSSs based on cisgenic designs represent a compelling candidate. Genomics of *C. hominivorax* now provides the foundation to translate decades of GSS research into this species, with validation of candidate genetic markers and the establishment of efficient HDR-mediated KI protocols representing key next steps. Among the approaches discussed here, the CGSS may offer important technical and regulatory advantages over other existing technologies, potentially facilitating their implementation within current SIT programs, and as such, should be considered a priority.

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Author Contributions

Daniel F. Paulo (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Writing—original draft [lead]), Omar S. Akbari (Conceptualization [supporting], Data curation [supporting], Formal analysis [supporting], Writing—review & editing [lead]), and Scott M. Geib (Conceptualization [supporting], Formal analysis [supporting], Writing—review & editing [supporting])

Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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Conflicts of Interest

The USDA is an equal opportunity provider and employer. The opinions expressed in this study do not imply endorsement by the USDA or the US Federal Government. OSA is a founder of Agragene, Inc., Synvect, Inc., and Cloak Biotechnologies with an equity interest. The terms of this arrangement have been reviewed and approved by the University of California San Diego, in accordance with its conflict of interest policies. All other authors declare no competing interests. During the preparation of this work, Grammarly was used to assist with language editing and readability. All authors critically reviewed and approved the final version of the manuscript.

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