Evaluation of genetic engineering and genome editing tools to develop multifactorial reproductive sterility or killing sperm systems for the improvement of the Sterile Insect Technique

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DECLARATION

I hereby declare that the doctoral thesis entitled

"*Evaluation of genetic engineering and genome editing tools to develop multifactorial reproductive sterility or killing sperm systems for the improvement of the Sterile Insect Technique*"

has been written independently and with no other sources and aids than quoted.

Für meine Familie

&

Paula

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1 Summary

Insects fulfill fundamental roles for the preservation of ecosystems such as pollination, but also represent key agricultural pests and human disease vectors accounting for immense economic losses and approximately one million deaths annually. While conventional strategies often entail environmentally damaging side effects and have failed to provide sustainable solutions to control insect pest and vector populations, the Sterile Insect Technique (SIT) has proven to be a powerful, species-specific and therefore environmentally sound tool in insect pest management. The principle of SIT is based on periodic inundative mass releases of male insects sterilized by ionizing irradiation, which results in infertile mating and ultimately in the decimation of the population. In order to mitigate the adverse effects of radiation on male fitness and competitiveness in classical SIT, and to enable efficient sex separation for male only releases, as well as to facilitate reliable monitoring by distinctive marking, several transgenic approaches have been devised and established in a variety of pest and vector species over the past twenty years. In addition, the use of engineered site-specific homing-based gene drives for insect pest control is currently heavily discussed.

Successful and efficient germline transformation remains a major obstacle and laborious task that aggravates the development of new and the transfer of existing transgenic SIT approaches in non-model pest and vector organisms. Therefore, we demonstrated, to the contrary of a previous publication, that employing helper plasmids encoding for a recently engineered hyperactive version of the most commonly used *piggyBac* transposase significantly enhances germline transformation rates in three different species of two different insect orders.

Moreover, I present my advances in the bioengineering of novel “killing-sperm” transgenic sterilization systems that could help to replace radiation in causing reproductive sterility. In a first approach, I started to bioengineer a killed-sperm system in the medfly *Ceratitis capitata* as an alternative approach to induce male sterility. However, the attempt to specifically kill the sperm has so far not been successful and needs further improvement on the time of expression or the use of genes causing apoptosis. In a second approach, I provide a perspective on using CRISPR/Cas in transgenic SIT to induce multifactorial sterility, which should be less sensitive to resistance development and therefore similar to irradiation-based approaches but specific to the sperm. In a third approach, I started to bioengineer a novel and innovative killer-sperm-based reproductive sterility system, in which males transfer along with their sperm a lethal factor that kills receiving females. Such a system should greatly improve SIT effectivity, as it not only guarantees male sterility but also restrains females from polyandrous mating and oviposition or blood sucking activities.

Furthermore, in respect to the use of site-specific homing-based gene drives for insect pest control, we generated a Cas9-based homing gene-drive element causing a female to male sex conversion in *D. melanogaster* and showed that non-homologous end joining increased the rate of mutagenesis at the target site. This resulted in the emergence of drive-resistant alleles and therefore curbed the gene drive, which indicates that simple homing CRISPR/Cas9 gene-drive designs will be ineffective.

2 Introduction

2.1 The impact of insects as agricultural pests and disease vectors

Insects (Insecta) represent by far the most diverse and species-rich class in the animal kingdom, amounting to more species than all remaining species combined. Today a total of approximately 1.5 million species have been named and described of which ~1 million belong to the class of Insecta (Costello et al., 2013; Zhang, 2011). Although previous hyper-estimations of up to thirty million existing insect species (Erwin, 1982) have recently been revised down to 2.6 to 7.8 million (mean: 5.5 million) (Stork et al., 2015), the identification of the vast majority of insect species is still pending (Ødegaard, 2000). Predominantly short generation times and a tremendous reproductive capacity are two key characteristics of insects responsible for their exceptionally fast ability to adapt to environmental changes, and thereby allowing them to populate almost every conceivable ecological niche, including hostile environments such as deserts and polar regions (Kelley et al., 2014; Pedigo and Rice, 2008).

However, precisely these properties in combination with proceeding global warming and human-related activities such as global trade and travel, and unbalanced monoculture farming systems have established the basis for the spread and infestation of many insect pest and disease vector species to additional areas and continents, posing a threat to agricultural commodities, livestock, human health and natural biodiversity (Bale et al., 2002; Epstein, 2001; Hulme, 2009; Tatem et al., 2006; Weaver and Lecuit, 2015; Wetzel et al., 2016).

Indeed, natural spreading of the majority of insect pest species barely exceeds distances of ten kilometers (Byrne, 2008), which was also demonstrated in a genetic population study of the Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Karsten et al., 2013). The Medfly originated from sub-Saharan Africa but can now be found in mild regions of almost every continent, including southern Europe (Figure 2-1) (Szyniszewska and Tatem, 2014). *Ceratitis spp.*, in conjunction with other species of Tephritid fruit fly genera, including *Anastrepha, Bactrocera,* and *Rhagoletis*, represent some of the world's most destructive agricultural pests, as females deposit their eggs in hundreds of varieties of healthy and ripening vegetables, fruits and nuts (Garcia, 2009). The emerging polyphagous larvae feed from the fruit tissue while the stinging-induced wound on the fruit additionally stimulates fungal and bacterial growth (Wimmer, 2005a). Consequently, affected fruits are not merchantable beyond local markets, causing annual economic losses of several billions of U.S. dollars, followed by imposed export bans to prevent further dispersal of the pest species (Malavasi et al., 1994; Oerke, 2006; Oliveira et al., 2014). This, however, is especially dramatic as agricultural exports are often the main source of income for fragile economies in developing and newly industrializing countries and embodies an additional problem to ensure food security.

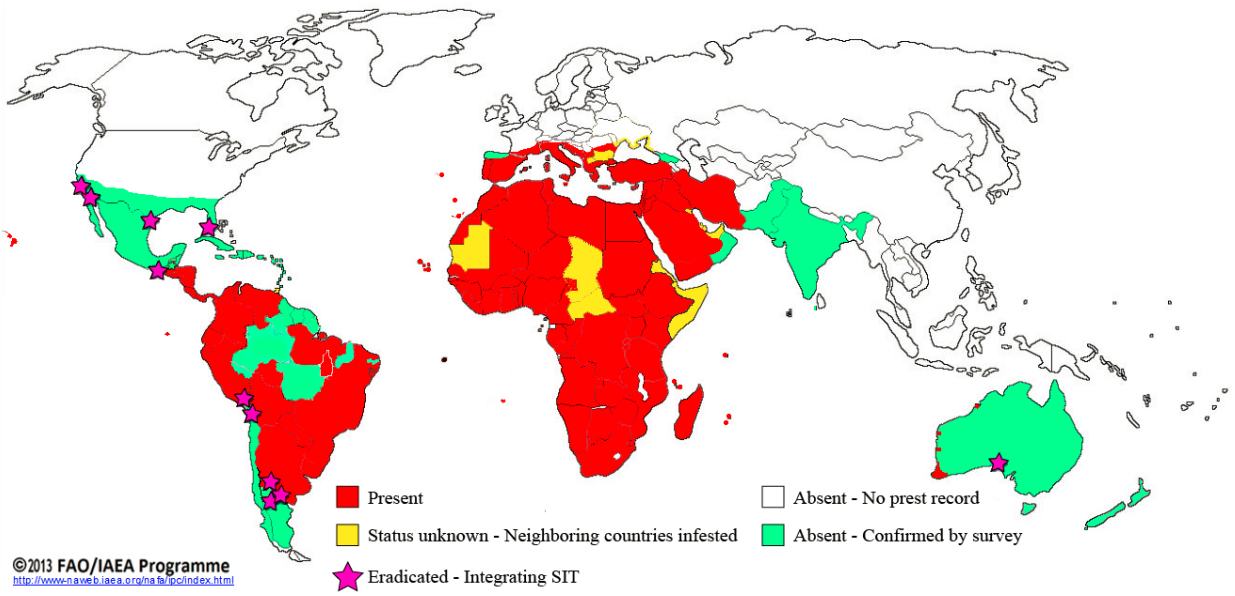

Figure 2-1 **Illustration of the worldwide distribution of *Ceratitis capitata* (status 2013)**
Red colored areas indicate presence of Medfly in country or geographic zone. In yellow marked areas the status of infestation is unknown, however, neighboring countries are infested. Land masses left in white are considered as pest-free with no record of Medfly. In green colored areas *C. capitata* is also absent, which is confirmed by survey. Pink stars show areas from which the Medfly was successfully eradicated, integrating SIT in pest management.
(Figure adapted from http://www-naweb.iaea.org/nafa/news/images; ©2013 FAO/IAEA Programme)

Insect-borne diseases such as Chagas disease, Chikungunya, Dengue fever, Malaria, Sleeping sickness, Yellow fever, and Zika fever, to name just the most prominent examples, lead to approximately one million of deaths every year (Wang et al., 2016; World Health Organization, 2014; World Health Organization and Global Malaria Programme, 2014). While the rate of Malaria infections is regressive, the cases of dengue have increased thirtyfold over the past fifty years with reported local transmissions increasing from initially nine to 128 countries, including recent cases in France, Croatia, the Madeira Islands of Portugal, and Florida, U.S.A. (Delisle et al., 2015; Succo et al., 2016; World Health Organization, 2014). Currently approximately four billion people are at risk of dengue, which equates to more than 50% of the entire world's population (Brady et al., 2012; Reiner et al., 2016). One crucial factor for this, similar to agricultural pests, is the alarming geographical spread that has repeatedly been reported for insect disease vectors with a special focus on the tiger mosquito *Aedes albopictus* due to its tolerance of colder temperatures (Kraemer et al., 2015; Schaffner and Mathis, 2014; Tatem et al., 2006). This is a particular cause for concern as *Ae. albopictus* is also a vector for other arboviruses (arthropod borne virus), including the Yellow fever virus, the West Nile virus, the Chikungunya virus and potentially the Zika virus (Amraoui et al., 2016; Bhatt et al., 2013; Semenza, 2015).

This fatal prevalence of vector-borne diseases on human health and animal farming as well as the detrimental effects of agricultural pests on economies and food security demonstrates the urgent demand for constant improvements to existing pest control strategies in combination with the development of new approaches to address present and future humanitarian challenges

2.2 Conventional control strategies and integrated management systems

Various control strategies have been developed and applied to counteract the adverse effects of pest and vector populations on agriculture and human well-being. Although most of the approaches were established and improved over the past decades, some of the fundamental ideas are as old as the practice of the cultivation of plants. The different sanitary and phytosanitary measures can be assigned to either of the four main principles of conventional control strategies encompassing *cultural control, biological control, physical and mechanical control* and *chemical control* (National Research Council, 1969).

Cultural and *biological control* represent the oldest groups of control strategies and are valued for their sustainability and ecological sensitivity. *Cultural control* is based on the avoidance of large monocultures and therefore promotes classical agricultural practices such as inter-cropping and crop rotation. For example, certain inter-cropped plants can act as a repellent or interfere with the pest's olfaction to impede host plant detection, whereas crop rotation can prevent the permanent infestation of an area if host plants are periodically not available (Mohamad Roff and Sivapragasam, 2005). Although very operative, cultural control tends to be limited to small scale farming (Liebman et al., 2001).

Conventional *biological control* was defined by DeBach (1964) as "the study and uses of parasites, predators and pathogens for the regulation of host (pest) densities". Predators and parasites, which include mites, parasitoid insects, nematodes and vertebrate species can be summarized as 'macrobial agents', whereas beneficial viruses, bacteria and fungi are grouped as 'microbial agents' (Bale et al., 2008; DeBach, 1964; Flint et al., 1998). However, besides reports of successful applications of conventional *biological control* (Gurr et al., 2000; Sweetman, 1935; Vincent et al., 2007), two opposite but major problems have been experienced: (i) the introduced species miscarries to establish a viable population, or (ii) the introduced species predominantly attacks non-target species and thus becomes an unmanageable invasive species, as was the case for the cane toad (*Bufo marinus*). This toxic anuran was released in Australia in 1935 to contain the greyback sugar cane beetle (*Dermolepida albohirtum*) but grew to one of the country's most severe invasive pest species with detrimental effects on the ecosystem (Aldhous, 2004; Brown et al., 2013; Phillips and Shine, 2004).

Other *biological control* measures, although not conventional, include genetic control, such as *birth control* strategies and *population replacement* approaches. *Birth control* attempts to suppress or eradicate a pest or vector population by impairing its reproductive capacity, whereas *population replacement* aims for the ingression of new traits (e.g. refractoriness to disease transmission) into the wild population and is therefore predominantly suitable for vector control purposes. Both strategies are based on the release of insects that can be engineered by physical, chemical, biotechnological or endosymbiont-

based means to introduce the desired properties described in detail in *2.3* and *2.4* (Bian et al., 2013; Dyck et al., 2005; Hammond et al., 2016; Wimmer, 2013).

Physical and mechanical control is comprised of rather obvious and straightforward measures which, with regard to vector control, involve the use of bed nets and window screens to prevent physical contact with disease transmitting organisms. Furthermore, the removal of breeding sites, such as tarns and ditches, are carried out with varying degrees of success (Sibanda et al., 2013). With respect to pest control, flytraps and sticky traps are regularly used, accompanied by fruit bagging or whole plant netting, and orchard sanitation. Yet, these measures are, similarly to *cultural control* strategies, restricted to rather small-scale productions (Ekesi and Lux, 2006; Vincent et al., 2001).

The most frequently used conventional control strategy is *chemical control,* largely because of the instant noticeable effects, even at high levels of acute infestation. In addition, synthetic insecticides often have a very broad spectrum of activity, such as Malathion and dichloro-diphenyl-trichloroethane (DDT) (Becker et al., 2010; Koul et al., 2008). However, this lack of specificity also brings about a multitude of adverse secondary actions, including the termination of beneficial, non-target insects, such as pollinators (Marzaro et al., 2011), parasitoids, and insectivore insects (Vilcinskas, 2011) that are essential for the overall integrity of an ecosystem, immunosuppressive effects on amphibians (Hayes et al., 2010), and cancerogenic effects on a variety of species including humans, particularly field workers exposed to high dosages of such chemicals (Loomis et al., 2015; Lynch et al., 2009). Furthermore, the extensive utilization of insecticides leads to resistance development in many insect species. This reduces the potency of the insecticide and requires the constant development of new substances, inflating production costs (Coleman et al., 2017; National Research Council, 1969).

Especially between the late 1940s and the mid-1960s control measures other than insecticide-based *chemical control* were largely neglected, wherefore this period is also referred to as the “Dark Ages” of pest control (Newsom, 1980). However, proceeding resistance development and the growing awareness of the harmful effects on ecosystems and human health has led to reevaluation of the *chemical control* approach and to the development of strategies such as the *integrated control concept* (ICC) (Stern et al., 1959). The ICC aims for a harmonious orchestration of several control strategies while confining the application of insecticides to a minimum for higher levels of sustainability and eco-friendliness, to set a new tendency in agro-entomology (Kogan, 1998). The subsequent extension of the ICC by economic, social, ecological and population dynamic deliberations added a necessary layer of management to the concept in general. This is now known as *integrated pest management* (IPM) (Bajwa and Kogan, 2002; Naranjo and Ellsworth, 2009; National Research Council, 1969). IPM programs usually start with an initial inventory of the entomologic state to identify the presence of one, or several pest species with due consideration given to the incidence of beneficial species. In a second step, an action threshold must be

determined that schedules which control measure should be put into action at certain, pre-defined infestation levels, while simultaneously emphasising preventative activities that keep pest densities low and avoid critical economic injury, and the need to use more invasive control measures (Kogan, 1998; Koul et al., 2008). A report about fresh grape production in California demonstrated that this line of action managed to reduce the use of broad-spectrum insecticides by 42% (Bentley, 2009). However, if the pest density exceeds the preassigned threshold, the complete repertoire of control techniques will be considered, including the use of insecticides as a last opportunity.

Analogous to IPM the coordinated control of disease vectors termed *integrated vector management* (IVM) was initiated and profited from the know-how gained in IPM systems. However, a number of adjustments to the framework had to be made including the involvement and commitment of central and local governments, local communities as well as the health-sector to work hand in hand with the coordinating united nation agencies (Beier et al., 2008; Challet, 1991; Lizzi et al., 2014; World Health Organization, 2004). Nevertheless, IPM has a considerable practical advantage over IVM, as the success can be directly measured in elevated crop yields. The achievements and economic impact of IVM are less apparent, which can strongly influence the long-term cooperativeness of local and governmental health authorities as well as private funding sources (Chanda et al., 2017; World Health Organization, 2004). Both, IPM and IVM, aim to consider the complete pest and vector situation with a focus on preventive and suppressive measures but are spatially restricted to individual farms or selected regions of abundant vector occurrence, respectively.

In contrast to this, the strategy of *area-wide integrated pest management* (AW-IPM) has been elaborated and emphasises the control of a key pest or a small group of pests over large areas, which adds to “prevention” and “suppression”, the possibility to eradicate a pest from a wider area (Faust, 2008; Knipling, 1978; Koul et al., 2008; Rabb, 1978). Today a key component of AW-IPM is the *birth control* strategy termed *sterile insect technique* (SIT) which was devised more than 60 years ago and opened a new era in ecologically sound pest and vector control (Dyck et al., 2005; Knipling, 1955; Wimmer, 2005a).

2.3 The sterile insect technique (SIT)

2.3.1 SIT principle and its significance in AW-IPM

The *sterile insect technique* (SIT) is a species-specific, environmental-friendly and self-limiting pest and vector control strategy. It is based on the principle of mass-releasing sterilized insects into a wild population that upon mating with wild-types generate unviable progeny and thereby diminish the overall reproductive success of the insect population, eliciting its decline (Krafsur, 1998). However, SIT is restricted to pest and vector species that can be mass-reared.

Although SIT is in most cases not a stand-alone technique, it emerged to one of the most valuable parts of today's AW-IPM programs for the control of various key pests and an increasing number of vector species since its conceptualization in the 1940s (Klassen and Curtis, 2005; Knipling, 1955). The groundwork for SIT was created with the early finding of x-ray induced sterility in tobacco beetle males (*Lasioderma serricorne*) in 1916 (Runner, 1916). This methodology was then transferred and further improved for the livestock parasite New World screw-worm fly (*Cochliomyia hominivorax*) (Bushland and Hopkins, 1951; Knipling, 1955; Lindquist, 1955), which resulted, after an effective initial area-test on the island of Curaçao (Baumhover et al., 1955), in the first successful SIT-based area-wide eradication program in the southeast of the U.S.A. and was later expanded to other areas, including the southwest of the U.S.A., Mexico, Panama (Knipling, 1960; Krafsur, 1998; Smith, 1960; Vargas-Terán et al., 2005). Other selected examples of prosperous eradication programs are the extirpation of the tsetse fly (*Glossina austeni*), the vector for Human African Trypanosomiasis (HAT, sleeping sickness), from the island Unguja (Zanzibar archipelago, Tanzania) (Vreysen et al., 2000), the painted apple moth (*Teia anartoides*), a polyphagous pest of horticulture, from New Zealand (Suckling et al., 2007), and the Medfly from northern Chile. In Chile alone, the outcome of this resulted in new trading opportunities for the fruit industry with an annual turnover of approximately 500 million U.S. dollars (Hendrichs et al., 2002).

In spite of these successful eradication programs, which consequently permitted the export of agricultural products to markets that demand a pest free status, apprehension that suppressive SIT strategies with a continuous release of sterile males would exceed reasonable costs and could not economically compete with other classical control measures resulted in SIT-based suppression control being largely barred from integration into AW-IPM programs (Hendrichs et al., 2005; Knipling, 1978). However, changes in public acceptance and imposed restrictions on the use of insecticides, new insights into their adverse effects, and a growing market for organic commodities (The Economist, 2001), together with a reduction in costs of mass-rearing insects, has made long-duration suppression strategies a cost-effective and attractive alternative to eradication programs. This was further strengthened as eradication

programs require a cost-intensive post monitoring phase and potentially additional containment measures to ensure that the pest free area is not re-infested. The probability of a reinvasion, which is among other factors influenced by the infestation status of neighboring areas, has to be taken into account when deliberating over whether eradication or suppression is the best option (Hendrichs et al., 2005). Suppression control generally accepts low levels of pest occurrence if the economic injury does not exceed a certain extent of tolerance. Food products from these areas are also suitable for export to markets that request pest free statuses if other risk-minimizing measures (e.g. physical plant protection) and/ or post-harvest treatments are conducted, which assure that there is no risk of pest dispersal (Cayol et al., 2004). Suppression programs were conducted, for example, for the Medfly in Hex River, South Africa (Barnes et al., 2004), on Madeira, Portugal (Pereira et al., 2000) and Neretva Valley, Croatia (Bjeliš et al., 2010) for the codling moth (*Cydia pomonella*) in British Columbia, Canada (Bloem et al., 1998; Calkins et al., 2000) and the oriental fruit fly (*Bactrocera dorsalis*) in Thailand (Enkerlin et al., 2003) and south India (Verghese and Mumford, 2010). However, with respect to the control of disease vector species or livestock pests, suppression strategies are seen as less appropriate.

Besides suppression and eradication strategies, the containment strategy can either serve as a measure to stabilize the improvements made during and after an eradication program or can be applied in infested areas to impede the dispersal of the pest or vector to other not yet or no longer contaminated regions and therefore acts as a shield. The strategy of prevention is essentially complementary to the containment strategy and is performed to maintain the pest or vector free status of an area and thus becomes especially important if adjacent areas are strongly infested (Hendrichs et al., 2005). To increase the effectiveness of containment and prevention measures, it was shown to be beneficial to consider strategic geographical features. For instance, as Panama's landmass is very narrow, the release of sterile screw-worm males effectively helps to reduce the migration of this livestock pest from infested South to pest-free Central and North America.

The main focus of SIT applications so far concentrated on agricultural pests, with a special emphasis on Tephritid fruit flies (Enkerlin, 2005; Klassen and Curtis, 2005). However, recent innovations in relation to mosquitos regarding the key steps necessary for the production and release of sterile males, will help make it possible to implement SIT strategies that successfully manage disease vectors (Benedict and Robinson, 2003; Bourtzis et al., 2016; Lees et al., 2015; Wilke et al., 2009).

2.3.2 Improvements of SIT

The success of an SIT program fundamentally relies on the generation of high quantities of sterile, fit males that upon release can effectively compete with wild-type males to reduce the reproductive potential of a pest or vector population. To be able to rate this performance, released males must be marked to facilitate their discrimination from wild-type males during post release field monitoring. To this end, five key steps consisting of mass-rearing, sex-separation, sterilization, marking and release are necessary and each step requires species-specific adjustments and optimizations (Dyck et al., 2005). Since the onset of SIT, scientists have made many efforts to overcome a multitude of problems and shortcomings for virtually every one of the key steps. Recent advances in genetic engineering tools and their availability have enabled biotechnological improvements to SIT through the generation of transgenic strains that carry efficient sex-separation-, sterilization- and/or marking-systems in various pest and vector species (Robinson et al., 2004; Schetelig and Wimmer, 2011). Several of the key improvements to SIT will be discussed below, without making the claim to be complete.

(i) Sex-separation

Although the principle of SIT aspires towards a male-only release, early SIT programs were constrained to conduct bisexual releases due to the unavailability of efficient sex-separation methods. This has led to increased costs of mass-rearing and an overall diminished effectiveness of SIT, as the released sterile males could also mate with co-released females instead of with the target wild-type females. In fact, a comparative study of bisexual versus male-only large-scale field releases of sterile *C. capitata* flies affirmed a several-fold higher effectiveness when releasing sterile males only (Rendón et al., 2004). Furthermore, independent of their sterility, it is the female mosquito that feeds from blood and thereby potentially transmits diseases as well as it is the female Tephritid fruit fly that causes a stinging-induced wound to the fruit, which promotes fungal and bacterial growth, even if no eggs are oviposited (Hendrichs et al., 1995; Wimmer, 2005a).

Initial efforts to separate sexes manually, for example, based on sexual dimorphisms in pupal-size or weight (Ansari et al., 1977) were labor intensive, entailed error rates of up to 15% and did not reduce mass-rearing expenses (Klassen and Curtis, 2005; Seawright et al., 1978). An initial improvement that disburdened the sex-separation during the mass-rearing of tsetse flies was made by considering sex differences regarding the generation time that enables male collection and female removal at discriminative eclosion time-points (Opiyo et al., 2000). Further sophistication in this respect was achieved with the development of *genetic sexing strains* (GSS) via classical genetics. The majority of GSSs in Tephritids carry a mutation that confers a visible phenotype, such as an altered pupal color, which

is only apparent in females, as males feature a rescuing wild-type allele translocated to their Y chromosome (Robinson, 2002a), facilitating photoelectric mechanical sorting of male and female pupae (McInnis et al., 2007). Such GSSs have been developed for several fruit fly species including the melon fly (*Bactrocera cucurbitae*) (McInnis et al., 2004), the oriental fruit fly (*Bactrocera dorsalis*) (Isasawin et al., 2013), the Mexican fruit fly (*Anastrepha ludens*) (Zepeda-Cisneros et al., 2014) and the carambola fruit fly (*Bactrocera carambolae*) (Isasawin et al., 2014). Nevertheless, such strains still require the rearing of female larvae. More elaborated GSSs carry a second conditional recessive lethal mutation combined with a Y-linked dominant rescuing allele that enables male survival while females die during embryonic stages. Following this, the *Vienna8* Medfly strain was generated by linking the mutant alleles *white pupae* (*wp*) (Rössler, 1979) and *temperature-sensitive lethal* (*tsl*) (Franz et al., 1996; Robinson, 2002b). Heat-shock applied to embryos of *Vienna8* effectively kills all females and enables selective male rearing (Franz, 2005). Systems developed for mosquito species were predominantly based on the translocation of an insecticide resistance gene to the Y allosome (Curtis et al., 1976; Lines and Curtis, 1985; Robinson, 1986). However, GSSs often take many years to be generated and were found to be genetically unstable due to occurring mutations and genetic recombination. They have a reduced level of fitness, and a system established in a certain pest or vector species cannot be easily transferred or adapted to another species (Gilles et al., 2014; Schetelig and Wimmer, 2011).

To overcome these limitations several biotechnological approaches for the generation of *transgenic sexing strains* (TSSs) have been genetically engineered. Many of those TSSs employ the *tetracycline repressible binary tTA expression system* (tet-off system) (Figure 2-2 & chapter 3.2.2) to conditionalize female-specific lethality, enabling stock-keeping and mass-rearing on a tetracycline-supplemented diet (permissive condition) (Freundlieb et al., 1999; Gossen and Bujard, 1992; Horn and Wimmer, 2003; Thomas et al., 2000).

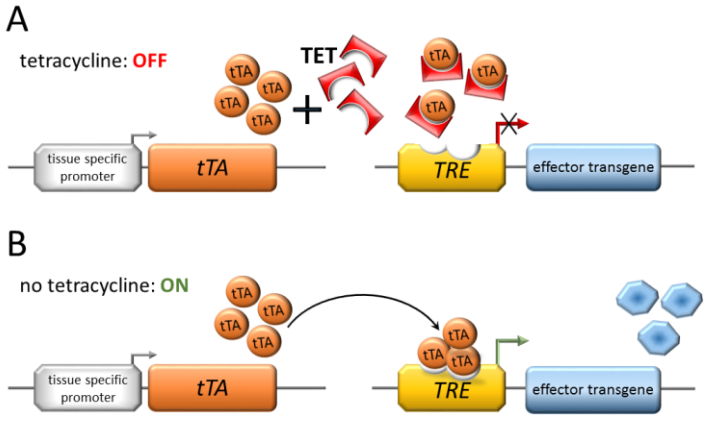

***Figure 2-2* Repressible binary tTA-system (tet-off-system)**
(A) Permissive/rearing condition**:** in the presence of tetracycline the tissue-specifically expressed tetracycline-repressible transactivator (tTA) is unable to bind to the tTA response element (TRE) and therefore cannot activate the transcription of the effector transgene (off-state).
(B) Restrictive/release condition: in the absence of tetracycline the system is on, since tTA is not detained from binding to the TRE and therefore can drive the expression of the effector transgene. (Figure adapted from Eckermann et al., 2014; see 3.2.2)

This is also the case for the first genetically engineered TSSs. Generated in *Drosophila melanogaster* as a proof-of-concept, these TSSs utilize regulatory regions of female-specifically expressed genes, such as the *Yolk protein 3* (*Yp3*) or *Yolk protein 1* (*Yp1*), to indirectly drive the transcription (via tTA/TRE) of a dominant lethal effector transgene (Heinrich and Scott, 2000; Thomas et al., 2000). However, the *Yp1* and *Yp3* enhancer were shown to initiate female lethality only at late larval, pupal or even early adult stages, which would again not reduce rearing costs. The same problem of a late onset of female elimination also applies to a sexing system developed by the company Oxitec (Abingdon, UK), which was first established in the Medfly (Fu et al., 2007), and was then, despite its shortcomings, transferred to the olive fruit fly (*Bactrocera oleae*) (Ant et al., 2012), the silkworm (*Bombyx mori*) (Tan et al., 2013), the pink bollworm (*Pectinophora gossypiella*) (Jin et al., 2013) and the New World screw-worm (Concha et al., 2016). This autocidal genetic system is comprised of a tTA positive feedback loop that eventually leads to the accumulation of lethal dosages of tTA (Gong et al., 2005). Although the mechanism is not fully understood, it is assumed that high levels of tTA tie up the transcriptional machinery, preventing the expression of essential genes. Restriction of this effect to females is accomplished by the incorporation of the sex-specifically, alternatively spliced first intron of the *C. capitata transformer* (*Cctra*) gene (Pane et al., 2002) into the *tTA* CDS. Only the female splice variant results in a translatable, full length ORF, whereas the male mature mRNA contains a premature stop codon, disabling tTA translation.

To avoid the necessity of rearing undesired female larvae, our laboratory has more recently engineered an early-acting *female-specific embryonic lethality* (FSEL) system in the Medfly (Ogaugwu et al., 2013). This system also employs the *Cctra* intron but has it integrated into the CDS of the phosphoacceptor-mutant allele hid^{Ala5} from *D. melanogaster* (Bergmann et al., 1998) that translates into a constitutively active version of the pro-apoptotic protein head involution defective (Horn and Wimmer, 2003). Expression of this TRE-based effector construct is timed exclusively to embryogenesis through the utilization of the regulatory region of an early cellularization gene that drives the transcription of tTA (Schetelig et al., 2009a), resulting in 100% female embryonic lethality. This FSEL system, also referred to as transgenic embryonic sexing system (TESS), was simultaneously developed in the Caribbean fruit fly (*Anastrepha suspensa*) (Schetelig and Handler, 2012a, 2012b) and recently established in the sheep blow fly, *Lucilia cuprina* (Yan and Scott, 2015), demonstrating its transferability.

A third TSS approach that makes use of a sex-specifically spliced intron has been developed for the three mosquito species *Ae. agypti* (Fu et al., 2010), *Ae. albopictus* (Labbé et al., 2012) and *Anopheles stephensi* (Marinotti et al., 2013), and is based on the female-specific conditional expression of a "lethal" effector transgene in the indirect flight muscle (IFM), which leads to flightless females, easing their

separation from males. However, this system cannot positively contribute towards a reduction of rearing expenses.

As a good alternative to female-specific lethality, Pane et al. (2002) and Salvemini et al. (2003) conducted an RNAi-based study in *C. capitata* that caused female-to-male conversion by knocking down *transformer* or *transformer-2*, respectively, essential genes for female fate, resulting in fertile XX males instead of females (Pane et al., 2002; Salvemini et al., 2003). However, the generation of a transgenic RNAi strain that includes conditionalizing this effect is still pending. A recent attempt to obtain a similar effect by the knockdown of *transformer-2* (*tra-2*) in *Ae. aegypti* was not effective due to differences in the sex-determination pathway but nevertheless induced high levels of zygotic female lethality (Hoang et al., 2016).

(ii) Sterilization

The sterilized insect represents the centerpiece of SIT and has been defined by the FAO as "an insect that, as a result of a specific treatment, is unable to reproduce" (FAO, 2005). Nevertheless, it is worth noting that in the sense of SIT the sterility of males does not necessarily implicate infertility. Indeed, sterile male insects generated by currently applied sterilization methods still produce sperm that are capable of fertilizing eggs, but carry dominant lethal mutations that impede their further development, which is referred to as "reproductive sterility" (Klassen and Curtis, 2005). Such mutations can either be induced physically, utilizing ionizing radiation, chemically, applying insect chemosterilants, or can be biotechnologically introduced as transgenes (Helinski et al., 2006).

Although chemosterilants have been shown to be very potent in terms of inducing sterility without severely affecting the fitness and competitiveness of treated male insects (Dame, 1985; Dame et al., 2009; Flint et al., 1975), their use for sterilization has been largely abandoned since the 1970s, as they were found to be carcinogenic and teratogenic, posing a serious hazard to the environment as well as facility-labor safety (Bakri et al., 2005; Campion, 1972; Hayes, 1968). Therefore, the employment of ionizing gamma radiation has prevailed as the standardized method and has since been applied to and adjusted for over 300 different arthropod species. The importance of careful adjustments becomes evident when considering that the absorbed radiation dose necessary to cause full sterility can vary between arthropod species by a factor of sixty (Bakri et al., 2005).

The underlying mechanism for sterility is based on radiation-induced chromosome breaks in gonial cells that persist in mature sperm. Post fertilization and syngamy (pronuclei fusion), during the first mitotic cell divisions, the chromosome breaks lead to the formation of dicentric chromosomes that cannot be separated properly during anaphase. This results in genetic imbalance due to chromosome aberrations, including aneuploidy and translocations, finally leading to the death of the embryo (Lachance, 1967;

Robinson, 2005; Smith and von Borstel, 1972). However, this effect is not restricted to the germline but also affects other stem-cell niches as well as somatic cells, which brings about a diminished overall fitness and competitiveness compared to wild-type males. Consequently, this demands the production and release of higher quantities of sterilized males, which significantly increases the costs and thus jeopardizes the affordability of an SIT program (Calkins and Parker, 2005; Parker and Mehta, 2007). This problem is especially relevant for lepidopteran pest species, as they are to some extent radio-resistant due to their holocentric-like chromosomal structure with diffuse centromeres, which enables the correct and loss-free replication and separation even of fragmented chromosomes. Accordingly, extremely high doses of radiation must be applied to induce sterility, which in turn weakens fitness and field performance (Carpenter et al., 2005; Horn and Wimmer, 2003; Pedigo, 1998).

To circumvent the negative effects linked to radiation-based induced sterility, transgenic systems have been genetically engineered to generate vigorous, yet reproductively sterile male insects. Such males pass on a conditional dominant lethal transgene combination that is ectopically expressed in the developing progeny and consequently causes lethality. These systems are also referred to as *release of insects carrying a dominant lethal* (RIDL) (Thomas et al., 2000). Similarly to the aforementioned TSSs, RIDL systems also utilize the binary tet-off-system to enable mass-rearing (Catteruccia et al., 2009). Pioneering work in this field was done by Horn and Wimmer (2003), who designed the first embryo-specific lethality system as a proof-of-principle in *D. melanogaster,* which was later successfully transferred to *C. capitata* (Schetelig et al., 2009a) and *A. suspensa* (Schetelig and Handler, 2012a),and furthermore provided the basis for the generation of the FSEL systems. Both, the *D. melanogaster* and the *C. capitata* RIDL strain comprise – analogous to the above-mentioned FSEL approach – a promoter/enhancer of a gene that is specifically expressed in the early embryo to drive, under restrictive conditions, the expression of the TRE-controlled $Dmhid^{Ala5}$ allele, killing all offspring at embryonic stage. In comparison to this, Oxitec developed late-acting dominant repressible lethality systems, which have been established for *C. capitata* (Gong et al., 2005) and *Ae. aegypti* (Phuc et al., 2007), and possess, apart from the *tra* intron, the same genetic elements as their female-specific lethality autoloop overexpression approach. Lethality in these latter systems launches only at late larval stages, which is disadvantageous in the case of the Medfly, as hatched larvae will still cause crop damage. However, in the case of mosquitos mathematical modelling suggests that delayed lethality could be beneficial, as transgenic larvae, which are already set to die, would compete for resources with wild-type larvae and thus impede their development (Atkinson et al., 2007; Phuc et al., 2007).

Another sterilization approach that has been engineered in *An. gambiae*, which was initially designed as a sex-separation system (2.3.4), makes use of the homing endonuclease (HE) (2.4.2.2, *i*) I-*Ppo*I from *Physarum polycephalum* (Muscarella et al., 1990; Windbichler et al., 2008). HEs are rare-

cutting enzymes due to their relatively long recognition sites, that are, when used in heterologous systems, commonly referred to as meganucleases (Burt, 2003; Deredec et al., 2008; Jasin, 1996). However, against all odds, *An. gambiae* possesses multiple I-*Ppo*I target sites exclusively on the X allosome within the ribosomal DNA (rDNA) repeats. Windbichler et al. (2008) exploited this coincidence and generated a strain that expresses I-*Ppo*I under the control of the 5' and 3' regulatory regions of the endogenous spermatogenesis-specific *β2tubulin* gene, leading to the fragmentation of the X chromosome in X-carrying sperm. However, as it turned out, a significant amount of stable I-*Ppo*I protein is also being transferred along with the sperm into the oocyte where it additionally shatters the maternally inherited X chromosome, resulting in complete embryo lethality. In a follow-up study, cage experiments demonstrated the capability of the system to reduce the reproductive potential of a population, but also revealed that I-*Ppo*I elicits fitness and competitiveness diminution to the transgenic males (Klein et al., 2012). Once again, the establishment of an affordable and reliable high-throughput sex-separation system for *An. gambiae* will be necessary in order to envisage this technique for future SIT programs.

(iii) Marking

After the release of the mass-reared sterile males, it is crucial to monitor their presence, survival, and dispersal in the release area, and estimate the sexual competitiveness with wild-type males (Vreysen, 2005). This is usually done with the *mark-release-recapture* (MRR) method, which requires reliable marking of the released males to enable their differentiation from wild-type males, and also necessitates the installation of traps to obtain a representative number of flies that reflects the actual population of flies present (Winskill et al., 2015). Conventionally, marking is achieved by dusting the pupae with a fluorescent dye that adheres to the insect's body during eclosion (Enkerlin et al., 1996). However, dyes are expensive, known to be hazardous to humans and natural enemies of the insects, and are error-prone – they can give false negative information when the dye has washed off or a false positive readout if the dye was transferred to wild-type males during flock-mating (Hagler and Jackson, 2001). Alternatively, phenotypic mutations could be utilized as markers but they are known to implicate fitness costs and cannot be transferred to other vector or pest species, similar to GSSs (Calkins and Parker, 2005; Niyazi et al., 2005). Furthermore, neither of the two marking strategies mentioned can provide information about the mating success of released males. So far this was only evaluated by comparing the sperm-head length of stored sperm in trapped mated wild females, as sperm-heads of irradiated males are slightly shorter than those of wild-type males (McInnis, 1993).

The first transgenic improvements to overcome the shortcomings of classical marking systems have been made by ubiquitous expression of a fluorescent protein in the Caribbean fruit fly (Handler and Harrell, 2001a), which was further advanced by a Y-linked integration of a similar construct, restricting

the expression to males (Schetelig and Handler, 2013a). This not only provides reliable male only marking but could additionally enable automated fluorescent-based sex-separation of embryos. Although with rather low throughput rates, which might not meet demands for mass rearing, advanced automated sorting was initially shown to be applicable to third instar larvae of a transgenic *Anopheles stephensi* strain, using the Complex Object Parametric Analyzer and Sorter (COPAS®, Union Biometrica) (Catteruccia et al., 2005; Gilles et al., 2014). This strain does not possess a Y-linked integration but employs the endogenous promoter of the spermatogenesis-specific *ß2-tubulin* gene for the expression of a fluorescent protein in the developing male testis. This system has been subsequently adapted to the mosquito species *Aedes aegypti* (Smith et al., 2007) and *Anopheles gambiae* (Marois et al., 2012) and was moreover transferred to several Tephritid species, including *C. capitata* (Scolari et al., 2008a), *A. suspensa* and *A. ludens* (Meza et al., 2014, 2011; Zimowska et al., 2009), as well as the cherry vinegar fly *Drosophila suzukii* (Ahmed et al., 2019). However, the motivation to implement this system into Tephritid species was solely based on its marking strength as it produces fluorescent marked sperm. This not only allows to distinguish between the released and wild-type males, but it also facilitates tracing the sperm in the spermatheca of captured inseminated wild-type females and thereby eases the assessment of the sexual competitiveness of SIT males (Scolari et al., 2008a).

2.3.3 Current limitations of SIT

Based on the constant advancements of genomic engineering tools and their utilization for various insect species in combination with continually growing knowledge gained through basic research for a better understanding of developmental processes, many of the initial obstacles of early SIT programs have been effectively solved. This makes SIT one of the strongest instruments for pest control and the fight against disease vectors. Nevertheless, SIT effectivity could still be improved, if certain limitations were addressed, which are discussed below.

One advantage of radiation-induced sterility over transgenic approaches is the immunity towards resistance development, as the mutations are generated randomly due to chromosome fragmentation. Therefore, the development of a genetic design that mimics the process of irradiation, as part of a ‘redundant killing’ strategy, could meet emerged environmental concerns and thereby pave the way for the formulation of a legal framework and mediate public acceptance for the release of transgenic insect strains (Eckermann et al., 2014; chapter 3.2.2).

Another limitation is that the majority, if not all, of the established transgenic sexing- and sterilization-systems rely on the same repressible binary expression system (tet-off-system), which can become problematic when two or more systems are to be combined in a single strain. Thus, deliberation

of novel molecular designs to implement other, possibly activatable, binary expression approaches could prevent system-interference. This would add a higher level of safety by creating independent systems, which complement to a 'redundant killing' strategy (Eckermann et al., 2014; chapter 3.2.2).

The established transgenic lethality systems have successfully addressed the fitness-related drawbacks inherited in radiation-based sterilization. Nonetheless, in female insects, including species of Tephritidae (Bertin et al., 2010), Culicidae (Helinski et al., 2012) and Lepidoptera (Torres-Vila et al., 2004), polyandry and sperm storage from multiple males is common. This behavior and physiology can dilute the effectiveness of SIT, as stored wild-type sperm will also contribute to fertilization. In addition, females can still cause crop damage or transmit diseases. Therefore, the development of a transgenic system that constrains female fecundity and/or longevity after copulation with a released bioengineered male would significantly improve SIT efficiency (3.2.3).

2.3.4 Alternative self-limiting genetic insect control strategies to SIT

As previously mentioned (2.3.2, *ii*), the main attribute of SIT is the mass-release of reproductively sterile male insects that produce unviable offspring when mating with wild-type females, which makes SIT the most rigorous self-limiting genetic insect control strategy, providing security that artificially introduced transgenes will not persist in wild populations. However, there are also several less stringent self-limiting genetic control approaches, which permit the temporary introgression of, for example, a sex ratio distorting transgene into a wild population that will vanish over the course of a limited number of generations.

One such strategy is termed *female-specific RIDL* (fsRIDL) (Thomas et al., 2000). This strategy aims for the direct release of homozygous males of the FSEL sex-separation strains, described in chapter 2.3.2, *i*. In fsRIDL approaches, sterility is selective and impedes the generation of female offspring, as released homozygous FSEL males will produce solely hemizygous FSEL sons, but no daughters, when mating with females of the wild population. In the next generation still half of the female progeny will die, whereas 50% of the male offspring will carry on inheriting the lethal construct and so on and so forth. This effect could be even enhanced, if the released males carried multiple copies of the fsRIDL construct on different chromosomes (Alphey, 2014; Schliekelman and Gould, 2000).

A very similar outcome can be acquired with the recently revised version of the I-*Ppo*I meganuclease-based X-shredder approach for *An. gambiae*, described in chapter 2.3.2, *ii*. In order to restrict embryonic lethality to females and obtain male offspring for release, and achieve successive inheritance of the transgene thereafter, Galizi et al. (2014) engineered an altered version of the I-*Ppo*I protein that possesses a reduced half-life. This destabilization of I-*Ppo*I confines its X-shredding activity

to spermatogenesis, as no functional I-*Ppo*I protein is dragged along with the sperm into the oocyte, preventing the eventual shattering of the maternally inherited X chromosome and facilitating the survival of XY male embryos (Galizi et al., 2014). This system was subsequently recreated utilizing the CRISPR/Cas9 system (2.4.2.2, *ii*) to accomplish DSBs-mediated X chromosome fragmentation during sperm development (Galizi et al., 2016). The advantage of the CRISPR-based approach over the I-*Ppo*I meganuclease method, is the ability for target site selection through guide RNA design, instead of being tied to the predefined recognition sites of I-*Ppo*I. This enabled targeting a different X-linked repetitive rDNA sequence that is specific to species of the *An. gambiae* complex, but is not conserved in more distantly related species, as it is the case for the I-*Ppo*I recognition site, addressing potential ecological concerns in case of the unlikely event of horizontal gene transfer.

2.3.5 Self-sustaining genetic insect control strategies

In the previous subchapters 2.3.2 and 2.3.4 several mating-based genetic insect control strategies have been described in which genetically engineered males pass on a dominant lethal transgenic construct to their progeny that kills either all descendants (RIDL), or only their female offspring, while their sons survive (fsRIDL). These approaches are collectively classified as self-limiting genetic control methods (Alphey, 2014). As in the case of RIDL systems, the transgene will not be transmitted into the population at all due to the absence of viable descendants. In the event of fsRIDL systems, the transgene will disappear rapidly from the population by natural selection, owing to the fatal fitness costs that it imposes to inheriting daughters, granddaughters, etcetera. Hence, periodic inundative releases are required to successfully contain or locally eradicate a pest or disease vector population when applying self-limiting population suppression strategies (Burt, 2014).

In contrast, self-sustaining genetic control strategies take a different, more invasive line of action. They aim at an enduring vertical transmission, persistence, and spread of a transgene in a target population, even if the modification-associated trait does not confer a benefit or yet entails a strong fitness penalty to certain carrier individuals and ultimately to the overall population (Alphey and Alphey, 2014). Depending on whether the dispersal of the transgene is intended to cause a decline, or a change of the genetic make-up of a target population to attenuate its vectorial capacities, such heritable approaches are classed as either population suppression or population replacement strategies, respectively (Burt, 2014). In virtue of the self-propagating quality, such strategies would require only a single or a few seeding release(s) of much smaller quantities of individuals. This could significantly reduce the costs of pest and vector management programs, but at the expense of their controllability. However, bioengineering of such heritable systems that are capable of bypassing natural selection and facilitating an overproportional dissemination of a

transgenic construct, detached from standard Mendelian inheritance, requires exceptional genetic mechanisms (Sinkins and Gould, 2006; Wimmer, 2013). In nature, various types of so-called "selfish" genetic elements (SGEs) have been discovered in all domains of life, which have evolved such mechanisms that are collectively termed as gene drives (Hurst and Werren, 2001; Lindholm and Price, 2016; Werren et al., 1988). Although these natural mechanisms usually differ substantially from one another, gene drives of SGEs identified in eukaryotic organisms can be categorized into three main concepts, namely: gonotaxis, interference, and overreplication (Burt and Trivers, 2008; Hurst et al., 1996).

Gonotaxis comprises a group of gene drives in which SGEs have developed mechanisms, that enable them to escape a terminating fate by ensuring to be moved, or actively move, into used germline cells, and thereby being transmitted disproportionally into the next generation for further spread (Burt and Trivers, 2008). One example of how this is being achieved, is manipulating the process of chromosome segregation during the asymmetric meiotic cell divisions of oogenesis in such a way, that the SGE-containing chromosome will increasingly segregate to the ovule but evades the polar bodies – a special form of gene drive also known as "meiotic drive" (Sandler and Novitski, 1957).

Opposed to this, interference-based gene drive mechanisms of SGEs typically act in a toxin-antidote-like fashion, which confers super-Mendelian inheritance by averting the transmission of the wild-type allele or imposing a disadvantage to offspring that do not inherit the SGE allele – for instance, by killing those 50% of the sperm or the progeny, lacking the SGE-bearing chromosome (Burt and Crisanti, 2018; Burt and Trivers, 2008). Such a drive system was detected in *Tribolium castaneum* and termed maternal effect dominant embryonic arrest (MEDEA), based on the observations that offspring of hemizygous MEDEA-females will only be viable if they inherit the MEDEA allele from either their mother or father (Beeman et al., 1992; Beeman and Friesen, 1999). Although the actual underlying factors of this system remain unknown, these observations allowed to draw the conclusion, that a maternally delivered lethal toxin-like factor and a zygotically expressed antidote must be tightly linked in a single *MEDEA* locus, which constitutes the SGE. Thus, such an interference drive mechanism ultimately leads to a replacement of the population, with all individuals eventually carrying the SGE at least in a heterozygous state. This has spawned the idea to engineer artificial MEDEA-like gene drive systems for population replacement-based insect control strategies, consisting of a designed *MEDEA* locus, which is equipped with cargo effector transgenes that, for example, render the insect refractory to pathogen transmission (Sinkins and Gould, 2006). With this end in mind, Chen et al. (2007) generated the first synthetic MEDEA-like gene drive as a proof-of-principle in *Drosophila melanogaster*. For the toxin component of the MEDEA-like SGE, Chen and colleagues employed an oogenesis-specific promoter to maternally express two microRNAs, designed to silence a maternally delivered mRNA that is essential for early embryonic development (Chen et al., 2007; Wimmer, 2013). The antidote component contains a

CDS, which encodes for a codon-modified version of the essential mRNA, invulnerable to the microRNAs and expressed under the control of an early zygotically active promoter.

Lastly, overreplication subsumes drive mechanisms where SGEs manage to increase their copy numbers in the genome, which also generates super-Mendelian inheritance (Burt and Trivers, 2008). The most prominent SGEs of this category are the class I and class II transposable elements. Class I retrotransposons contain several genes, which encode for proteins, including a reverse transcriptase (RNA-dependent DNA-polymerase) and an integrase (2.4.2.1), that permit them to copy and paste themselves into new genomic loci (Bourque et al., 2018). Class II DNA transposons (2.4.1), on the other hand, typically encode for only a single protein, termed transposase. This enzyme is capable of excising its own corresponding DNA sequence from a present chromosomal location and subsequently re-integrating it into a new genomic locus, which often leaves a double-strand break (DSB) at the excision site. This alone does not lead to a multiplication of the copy number of the transposable element. However, timing this event in the germline to the 4N-stage after DNA replication, can lead to repair of the DSB through homology directed repair (HDR) (2.4.2.2; Hurst and Werren, 2001). Here, the sister chromatid – that still contains a copy of the transposable element at the initial locus – serves as repair template, resulting in a duplication of the SGE, whereas the previously excised transposon can integrate into a new locus. Another group of SGEs that also leverage HDR to attain overreplication, are homing endonucleases genes (HEGs), which encode for homing endonucleases (HEs), also known as meganucleases (chapters 2.3.2 & *ii*, 2.4.2.2, *i*; Burt and Trivers, 2008; Paques and Duchateau, 2007). This class of endonuclease enzymes creates DSBs at very specific and usually unique sites in the host's genome, resultant from their long recognition sequences. HEGs are typically situated at their own target sequence that is also present at the corresponding locus on the homologous chromosome (Burt and Koufopanou, 2004). Therefore, cleavage of the DNA at the vacant recognition sequence by the respective HE, results in an HDR-based copying of the HEG-bearing homing allele to the homologous chromosome, changing zygosity from hemi- to homozygous. This process is then referred to as homing (Stoddard, 2005).

Overreplication-based gene drive mechanisms have also long been envisaged for exploitation in self-sustaining genetic pest and vector control (Burt, 2003), with an initial emphasis on transposable elements (Beerntsen et al., 2000; Ribeiro and Kidwell, 1994). However, while valuable for insect transgenesis (2.4.1), their proposed use as vehicles for gene drive systems in genetic insect management has been largely withdrawn, as they have been found to spread frequently between species via horizontal gene transfer (Bartolomé et al., 2009; Jordan et al., 1999; Sormacheva et al., 2012), inherently possess elevated mutation frequencies, which limit their own lifespan and consequently would decrease transgene stability (Petrov et al., 1996; Spielman et al., 2002), and, first and foremost, are difficult to control regarding the location of genomic insertion and copy numbers (Braig and Yan, 2002; Burt, 2003).

Therefore, HEGs have been considered to be the more appropriate and controllable kind of SGE for the development of novel self-sustaining approaches, as their sequence-specific mechanism has a simpler and more predictable mode of operation (Burt, 2003). From this, several strategies leveraging HEG-mediated homing for population replacement and suppression approaches have been conceptualized, which, however, all start from the premise that the target specificity of HEs can be reprogrammed in order to recognize chosen sequences. In respect thereof, Burt (2003) proposed that a suppression gene drive could be feasible if an engineered HEG would be placed into and programmed to target a mutual sequence of a gene that is essential for female-specific viability or fecundity. In addition, HEG expression and, thus, HE activity should be restricted to the germline to facilitate super-Mendelian inheritance of the HEG-bearing knockout allele, while at the same time enabling an unimpaired development of hemizygous carrier females. Consequently, such females as well as hemizygous and homozygous drive-males would rapidly increase in numbers over the course of few generations, which, depending on the target gene, should eventually cause a population collapse, due to the lack of viable or fertile females. In an analogous manner, this principle design could also be put to use for replacement strategies, if the gene to-be-disrupted is non-essential for the insect itself, but is required for pathogen reproduction, development, or transmission, for instance. Moreover, and similar to the synthetic MEDEA system, such homing alleles could be designed to contain, and thereby introgress a cargo of anti-pathogen effector transgenes into a wild population, which reduce the insects' competence to serve as a host or vector.

The first study that demonstrated the practicability of creating HEG-based gene drives was performed in *Anopheles gambiae*, employing the natural HEG I-*Sce*I from *Saccharomyces cerevisiae*, which showed sequence-specific homing into an artificially introduced cognate I-*Sce*I recognition site at the corresponding locus on the homologous chromosome (Windbichler et al., 2011). Unfortunately, methods to readily reprogram the target specificity of natural HEs without affecting their catalytic properties have not materialized (chapter 2.4.2.2, *i*; Chan et al., 2013b). Therefore, attempts have been made to develop synthetic selfish elements exploiting the chimeric programmable protein-guided zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (2.4.2.2, *i*) (Simoni et al., 2014). However, these systems were found to frequently generate dysfunctional homing products that have lost their ability to spread. With regard to TALENs, this most probably results from the high amount of identical sequences in their modular building blocks that aggravate the recombination-based homing process. Only with the recent emergence of the CRISPR/Cas technology new possibilities have opened up to bioengineer artificial homing elements that can easily be designed to efficiently target and faithfully home into arbitrary genomic sequences, owing to the simple guide RNA-based programmability of the Cas endonuclease (chapter 2.4.2.2, *ii*; Esvelt et al., 2014; Gantz and Bier, 2015). In order for a CRISPR/Cas homing element (CHE) to function as a homing CRISPR/Cas gene drive (HCGD) system, it

must, at a minimum, consists of a construct that comprises a *cas* gene and a guide RNA (gRNA) including appropriate promoter sequences, which is incorporated into the genome at the gRNA's own target sequence. The first such HCGD system was established as a proof-of-concept in *Drosophila melanogaster* for which the CHE was placed into and designed to target the X-linked *yellow* (*y*) locus (Gantz and Bier, 2015). In order to obtain drive activity in both somatic and germline cells, the *vasa* promoter was utilized to express the *cas9* gene. Expectedly, crosses of hemizygous females carrying the CHE allele (y^{CHE}) to wild-type males resulted in high percentages of progeny that showed either a full body or mosaic *y*-phenotype of a yellow colored adult cuticle, indicating that the paternal wild-type *y* allele was successfully targeted and knocked out. However, the different possible events – whether based on the intended HDR or undesired NHEJ – were not addressed in this study. In a follow-up study, a similar CHE-design was used for the development of a Cas9-based population replacement drive in the malaria vector *Anopheles stephensi* (Gantz et al., 2015). Besides the autonomous gene drive elements necessary for homing, a cargo of two effector transgenes encoding for anti-*Plasmodium falciparum* ookinete and sporozoite antibodies were added to the CHE for spread into a laboratory *An. stephensi* population. In addition to that, over the past two decades, a whole range of promising antiparasitic effectors that could potentially be employed as cargo in replacement drives have been identified (Ito et al., 2002; James, 2003; Moreira et al., 2002; Wang and Jacobs-Lorena, 2013). These include, for instance, additional antibodies that recognize surface proteins of different *Plasmodium* stages (Isaacs et al., 2012, 2011; Santoyo and Romero, 2005; Yoshida et al., 2001), components of the mosquito's insulin signaling pathway (Corby-Harris et al., 2010), small proteins of the mosquito's innate immune system (Kim et al., 2004; Kokoza et al., 2010; Vizioli et al., 2001), and synthetic peptides, such as the synthetic dodecapeptide salivary gland- and midgut-binding peptide 1 (SM1) (Ghosh et al., 2001). The latter has previously been shown to effectively block *Plasmodium*-epithelium interaction in the mosquito's midgut and salivary glands, impairing the development and transmission of *Plasmodium* (Ito et al., 2002).

2.4 Genetic engineering and genome editing tools for pest and vector control

The ability to stably integrate exogenous DNA into an insect genome was one of the most important achievements for modern forward and reverse genetics. This opened up new ways to study gene-expression and -function, and was game-changing for the improvement of biotechnological applications, including transgenic pest and vector control (Wimmer, 2003). However, successful germline transformation has come a long way and initial transformation efforts to simply soak insect embryos or larvae in genomic DNA solutions were mostly abortive (Caspari and Nawa, 1965; Fox and Yoon, 1966; Handler and O'Brochta, 2011; Nawa and Yamada, 1968), as effective *in vivo* methods, genetic vector systems and reliable insertion markers had not yet been developed (Schetelig and Wimmer, 2011). The first transfer of recombinant DNA into a metazoan genome was achieved in *D. melanogaster,* using the transposable element *P* (Spradling and Rubin, 1982). Unfortunately, it quickly turned out that the functionality of this system depends on host-specific co-factors, impeding the adaptation of this technique to non-*Drosophilid* species (Handler et al., 1993; O'Brochta and Atkinson, 1996; Rio and Rubin, 1988).

Today, germline transformation protocols exist for a huge variety of invertebrate and vertebrate species, employing different molecular genome-modifying systems that have been developed over the past two decades. Other than transposon-mediated random integration (2.4.1), these include also several site-specific approaches such as tyrosine- or serine-catalyzed recombinases (2.4.2.1) as well as protein- or RNA-guided programmable endonucleases (2.4.2.2) (Fraser, 2012). Furthermore, universal fluorescent transformation marker systems have been invented, which confer a dominant neomorphic phenotype for the straight forward identification of transgenic individuals (Berghammer et al., 1999; Handler and Harrell, 2001a; Higgs and Lewis, 2000; Horn et al., 2000, 2002; Horn and Wimmer, 2000; O'Brochta and Handler, 2008). Nevertheless, the decision as to which of the different genome modifying systems should come into operation usually depends on several individual factors. This can include the availability of reliable sequence information and genome annotations (e.g. model versus non-model organisms), the actual necessity to target a specific sequence in the genome, the existence of pre-evaluated genomic loci or established landing sites, and can be in some cases merely a consideration between effort required versus benefit gained.

2.4.1 DNA transposon-mediated random integration for genetic engineering

Transposons, also known as transposable elements (TEs) or "jumping genes", are mobile selfish genetic elements, which can be subdivided into Class I RNA transposons (retrotransposons) and Class II DNA transposons based on the differential mechanisms of genomic locomotion. While retrotransposons facilitate their transposition via a "copy-and-paste-like" mechanism that involves an RNA intermediate and its reverse transcription (Finnegan, 2012), genomic movements of DNA transposons take place on a DNA-to-DNA level and can be exemplified as a "cut-and-paste-like" mechanism (Handler and O'Brochta, 2011). However, only the latter are employed for vector systems in insect germline transformation (Burns, 2000; Schetelig and Wimmer, 2011). Autonomous DNA TEs are composed of a transcription unit that encodes for a transposase, flanked by *inverted terminal repeats* (*ITRs*). The transposase protein specifically recognizes the *ITRs* and catalysis its own excision and insertion reaction (Muñoz-López and García-Pérez, 2010). The construction of non-autonomous, two component vector systems for transgene transfer makes use of this mechanism as any DNA fragment – however limited in size – that is framed by the *ITRs* can be translocated, if the respective transposase is supplied *in trans*, either by the co-injection of mRNA, protein, or expressed from a transposase encoding helper plasmid (O'Brochta et al., 2014).

Since the ascertainment of the aforementioned restrictions regarding the *P* element (2.4), numerous other metazoan TEs have been identified to date. Today, the genetic transformation of insects, including economically and medically relevant species, mainly relies on TEs of three transposon-families: the *Tc1/mariner* family, the *hobo/Ac/Tam3* (*hAT*) family, and the *TTAA*-specific family (Atkinson and O'Brochta, 2000; Fraser, 2000; Lampe et al., 2000). The *Mariner* element *Mos1* from *Drosophila mauritiana* (Bryan et al., 1987; Medhora et al., 1988) and the *Mariner*-like element *Minos* isolated from *Drosophila hydei* (Franz and Savakis, 1991) belong to the *Tc1/mariner* family (Plasterk, 1996; Plasterk et al., 1999). *Mos1* is mainly used in *Drosophilids* and *Ae. aegypti* (Coates et al., 1998; Garza et al., 1991; Lohe and Hartl, 1996), whereas *Minos* has been shown to be functional in several dipteran, coleopteran and lepidopteran pest species, including the olive fruit fly (Koukidou et al., 2006), the Medfly (Loukeris et al., 1995), the red flour beetle (*Tribolium castaneum*) (Pavlopoulos et al., 2004), the coffee berry borer (*Hypothenemus hampei*) (Acevedo et al., 2012), and the silkworm (Uchino et al., 2007). From the *hAT* family, *Hermes* is the most utilized transposon, which was discovered in *Musca domestica* (Atkinson et al., 1993; O'Brochta and Atkinson, 1996) and has been successfully applied in beetle, mosquito, fly and butterfly species (Allen et al., 2001; Berghammer et al., 1999; Marcus et al., 2004; Michel et al., 2001). However, in *Ae. aegypti* cases of non-canonical integrations have been detected, which contained larger parts of the donor plasmid's backbone (Handler, 2002a; Jasinskiene et al., 2000). The most widely-used mobile element is the *TTAA*-specific *piggyBac* transposon, which was isolated from a mutant Baculovirus

strain in *Trichoplusia ni* (Cary et al., 1989; Fraser et al., 1983). The *piggyBac* transposase (PBase) is exceptional for its immense spectrum of targetable species, ranging from flatworms to mammals, with reported successful germline transformation in 39 insect species from five different insect orders alone (Genç et al., 2016; O'Brochta et al., 2014; Schetelig and Handler, 2013b).

However, despite the general ability to generate transgenic strains in many different species, the actual rate of transformation has frequently been reported to be low, rendering germline transformation experiments laborious and inefficient. Therefore, several approaches have been carried out, regarding expression, translation and enzymatic performance of transposases, which effectively improved the capability of genetic vector systems. The utilization of endogenous heat-shock or constitutively active promoter/enhancer elements of the target species for an efficient expression of the transposase has been repeatedly shown as an effective measure to increase transformation events (Dippel, 2016; Handler and Harrell, 1999; Li et al., 2001). Furthermore, mammalian systems significantly profited from customizing the codon-usage of insect codon-based transposases to mammalian codon-optimized versions, enabling reliable translation of the transposase transcript (Cadiñanos and Bradley, 2007; de Wit et al., 2010). Another strategy proven to be effective is the engineering of hyperactive transposase versions based on site-specific mutagenesis or random mutagenesis screens. This has been successfully demonstrated for the *Tc1/mariner* transposons *sleeping beauty* (Geurts et al., 2003; Mátés et al., 2009; Zayed et al., 2004) and *Mos1* (Pledger and Coates, 2005), and more recently for the *piggyBac* transposase (Yusa et al., 2011). The hyperactive *piggyBac* transposase was made available with the original insect-based codon-usage (^{i}*hyPBase*) as well as in a mammalian codon-optimized version (^{m}*hyPBase*), which both showed considerably increased transposition rates in several mammalian *in vivo* and *in vitro* systems (Burnight et al., 2012; Doherty et al., 2012; Yusa et al., 2011). These findings were congruent with experiences made in our laboratory, employing ^{m}*hyPBase* encoding helper plasmids for germline transformation in *Ceratitis capitata* and *Tribolium castaneum* (Ogaugwu and Dippel, personal communication). Contradictory to these findings, a study conducted in *D. melanogaster* and *Ae. aegypti* utilizing ^{i}*hyPBase* reported on sterility effects and low transformation rates (Wright et al., 2013). This discrepancy appears somewhat astonishing, since both, ^{m}*hyPBase* and ^{i}*hyPBase*, encode for the same hyPBase protein. Therefore, we planned a systematic comparison of the two different *hyPBase* coding sequences for germline transformation in several insect species to shed light on these inconsistent results (3.1).

Although, the general mode of operation of all DNA transposons appear to be very similar, they can exhibit a number of differences with respect to DNA-cargo size limitations, target site specificity, and favored genomic loci for integration. For example, the *piggyBac* transposase invariably inserts into the canonical *TTAA* tetranucleotide target site with a bias towards the first intron of transcription units (Häcker et al., 2003; Thibault et al., 2004). In contrast to other transposases, which show reduced transposition

rates for cargos bigger than 10 kb (Zayed et al., 2004), the *piggyBac* transposase does not seem to exhibit such a limitation and was shown to be capable of mobilizing DNA fragments larger than 100 kb (M. A. Li et al., 2011). The 8 bp target site of the *P*-element transposase is more structural as opposed to being of a sequence-specific nature, with a preference for transposing into 5' regulatory regions – especially origins of replication (Liao et al., 2000; Spradling et al., 2011, 1995). *Hermes*, however, predominantly integrates into nucleosome-free, *actin5C* promoter-like sequences (Gangadharan et al., 2010; Guimond et al., 2003), whereas *Minos* does not seem to possess a precedence for a certain chromosomal location (Bellen et al., 2011). The occurrence of such transposon-specific "hotspots" can be due to sequence preferences, interactions with particular proteins (e.g. host factors) or can be influenced by the chromatin status of the DNA (Gangadharan et al., 2010).

However, the genomic position of the transgene can have severe implications on the functionality and fitness of the resulting transgenic strain. Position effects caused by "proximate" *cis*-regulatory DNA elements and/or the surrounding chromatin can dramatically alter transgene expression, leading, in extreme cases, to complete transcriptional silencing or an undesired "leaky" expression (Wilson et al., 1990; Wimmer, 2005b). The latter is of special concern when generating transgenic strains that carry a dominant lethal effector construct whose ectopic expression should be tightly regulated to a certain developmental stage and/or tissue. The utilization of insulator sequences has been shown to be effective to confine such influences (Chung et al., 1993; Horn and Wimmer, 2003; Sarkar et al., 2006). Inversely, the insertion of a transgenic construct into a regulatory or coding region (gene disruption) of an essential gene can dramatically impede the fitness and viability of the genetically modified organism.

Another inherent risk of transposon-based insertions is transgene instability due to re-mobilization events. These can lead to a complete loss of the transgene, its re-integration into a less suitable genomic position or enable its horizontal spread into genomes of other organisms, raising ecological concerns for release programs (Schetelig et al., 2011). Remobilization may occur if the *ITR*-embedded transgene construct is re-exposed to either its corresponding or a related cross-acting transposase present in the host species' genome. This ability of related TEs to cause cross-mobilization was previously confirmed between the *hAT* elements *hobo* and *Hermes* (Atkinson et al., 1993; Sundararajan et al., 1999). Additionally, also *piggyBac*-like elements were identified in various species of the three eukaryotic kingdoms of Animalia, Fungi and Plantae, including species relevant for pest control, such as *B. dorsalis* (Handler, 2002b; Sarkar et al., 2006), which demonstrates that TEs are frequently transmitted horizontally.

To ensure that an integrated transgene of a positively evaluated strain retains its genomic location, it is necessary to render the insertion inert to possible exposure to cross-acting transposases. To this end, several methods have been developed that aim for either the removal or rearrangement of *ITRs* (Handler, 2004). The deletion of one or both *ITRs* can be achieved, if the primarily inserted transposon of the donor

vector contains additional *ITRs* besides the regular pair necessary for initial integration, subdividing the comprehensive transposon into several possible "sub-transposons" that each contain a distinct marker or combination of markers. Successfully transformed F1 flies should thus express the complete set of markers. Re-exposure of such isolated F1 flies to the respective transposase – by either crossing the transgene carrying strain to a transposase expressing "jumpstarter" strain (Horn et al., 2003) or re-injecting the helper plasmid, mRNA or protein of the transposase – enables the mobilization and consequential excision of the undesired sub-transposon(s). Flies with only one or no *ITR* remaining can be identified in the next generation by selective screening for individuals that exclusively express the marker(s) of the desired subunit. The single *ITR* deletion strategy was first realized in *Drosophila melanogaster* (Handler et al., 2004) and successfully transferred to the Tephritid species *Anastrepha ludens* (Meza et al., 2011) and *Anastrepha suspensa* (Handler and Schetelig, 2014). The approach that excises all *ITRs* was established directly in *Ceratitis capitata* (Dafa'alla et al., 2006). Other approaches that allow post-integration modification to remove or rearrange *ITRs* are based on the deployment of site-specific recombination systems such as Cre/*loxP*, Flp/*FRT* or ΦC31-Int/*att* (2.4.2.1) given that the initial construct comprises the required recombinase recognition target site(s) (Horn and Handler, 2005; Schetelig et al., 2011, 2009b).

2.4.2 Site-specific genetic engineering and genome editing tools

2.4.2.1 DNA recombinase systems for site-specific genetic engineering

(i) Constituents, mechanisms and types of DNA rearrangements

Site-specific recombination is a process of DNA rearrangements resulting from cleavage and subsequent reciprocal reunion of strands at two defined recognition target sites (RTSs) catalyzed by homodimers or several monomers of the respective recombinase protein. The majority of site-specific recombinase (SSR) systems derive from bacteriophages or selfish genetic elements (2.3.5), which evolved this type of recombination mechanism to be capable of using the host's replication machinery for the amplification of their own DNA. For example, in case of a temperate phage: the integration of its genome into the host's chromosome to enter the lysogenic state (Grindley et al., 2006; Gupta et al., 2007; Stark, 2015). Depending on the catalytic amino acid (aa) serving as nucleophile in the enzyme's active site, most site-specific recombinases can be assigned to either of the two major recombinase families: the tyrosine (Tyr) or the serine (Ser) recombinase family (Lee and Sadowski, 2003; Turan and Bode, 2011). Well-studied and commonly used SSRs are the Tyr recombinase Cre (causes recombination) from phage P^1 of *Escherichia coli* (Rizvi et al., 2018; Siegal and Hartl, 1996; Sternberg and Hamilton, 1981) and Flp

(flippase) from the 2 µm plasmid that resides in the nucleus of *Saccharomyces cerevisiae* (Broach and Hicks, 1980; Futcher, 1986). The most widely utilized Ser SSR is the ΦC31-Int (integrase ΦC31) from phage ΦC31 of *Streptomyces spp.* (Groth et al., 2004; Kuhstoss and Rao, 1991).

Conservative site-specific recombination is mediated between two recombinase-specific RTSs, which, depending on the recombinase, can be identical or exhibit sequence variations to some degree. Although the complexity of an RTS can also vary between recombinases, commonly used SSR systems are operative with only minimal RTSs. These are typically made-up of a short stretch of DNA (<50 bp) that contains two recombinase binding elements (RBEs) (armL and armR), flanking a central crossover region (spacer). The spacer not only predefines the position of the single-strand breaks (SSBs) at its 5' margins but additionally imparts the general directionality of the RTS due to its usually asymmetric sequence (Craig et al., 2015; Haenebalcke and Haigh, 2013; Turan and Bode, 2011). As in the case of Cre and Flp, two identical *loxP* (locus of crossing over (x), P1) sites or *FRT* (Flp recognition target) sites are recombined, respectively, the RTSs are regenerated, making the reaction reversible and thus bidirectional (García-Otín and Guillou, 2006). In contrast, the ΦC31-Int recombines the two slightly different attachment-sites (*att*-sites) *attP* (phage attachment site) and *attB* (bacterial attachment site), which leads to the formation of incompatible *attL* and *attR* hybrid product sites. In this instance, the reverse reaction would require the addition of the ΦC31-specific recombination directionality factor (RDF) gp3. In the absence of gp3 the recombination reaction is consequently unidirectional (Farruggio et al., 2012; Khaleel et al., 2011).

A site-specific single-recombination between two respective RTSs can potentially lead to either an *(i)* integration (fusion), *(ii)* translocation, *(iii)* excision (resolution, deletion), or *(iv)* inversion of DNA. However, the actual mediated type of DNA rearrangement depends on the relative orientation of the RTSs to one another and whether the RTSs are in *cis* or in *trans*. *(i)* A recombination between two RTSs that are located in *trans* with at least one of the DNA molecules being circular (e.g. a plasmid) results in the integration of the circular into the linear DNA molecule. If both DNA molecules are circular, the process is termed fusion. The relative orientation of the RTSs determines the overall orientation of the insertion. *(ii)* In a similar scenario, however, where both DNA molecules are linear, an exchange of the flanking DNA takes place, which is then called translocation. Yet again, the relative orientation of the RTSs must be considered as, for example, in the case of inter-chromosomal translocations, only recombination of equally oriented RTSs (with regards to the centromere) leads to the chromosomal arm exchange. Recombined RTSs of opposite orientations leads to the creation of undesired di- and acentric chromosomes. *(iii)* Intramolecular excision of a DNA segment takes place if two *cis*-linked RTSs recombine that are of the same orientation (head-to-tail). *(iv)* In contrast, a segment flanked by two oppositely oriented RTSs (head-to-head or tail-to-tail) will be inverted after recombination (Craig et al.,

2015; Haenebalcke and Haigh, 2013; Langer et al., 2002; Ow and Medberry, 1995; Stark, 2015; Turan and Bode, 2011).

(ii) Applications of SSR systems and the development of new SSR-based techniques

The deployment of SSR systems and ensuing diverse opportunities for site-specific genome modifications that function in prokaryotes as well as eukaryotes *in vivo* and *in vitro*, allowed for new means with which to address prevailing limitations and problems in basic research and several branches of biotechnology. A substantial advancement in this regard, was the novel ability to spatially and temporally restrict homozygosity of mutant alleles to, for example, a certain cell type, tissue or developmental stage, by making use of the inherent binary property of SSR systems via conditional recombinase expression. This gave developmental biologists new means to perform loss-of-function experiments of genes in adult animals that are also essential during early development (Dang and Perrimon, 1992; Sauer, 1994; Theodosiou and Xu, 1998). With this in mind, advanced SSR-mediated mitotic recombination systems for clonal analysis, such as the mosaic analysis with a repressible cell marker (MARCM) system, have greatly eased the generation, identification and investigation of marked homozygous mutant cells (Blair, 2003; Lee and Luo, 1999). Moreover, SSR systems formed the basis for the development of the "Invitrogen™ Gateway™ recombination cloning technology" (Katzen, 2007), were furthermore suggested for use in gene therapy (Karow and Calos, 2011; Scott et al., 2000), and have been established as genetic engineering tools for site-specific transgene integration. The latter in particular was a pivotal achievement for the generation of bioengineered insects, as it enables the insertion of transgenic constructs into specific, pre-evaluated loci that are known to lack position effects (2.4.1 & 3.1). In this respect, the ΦC31-Int/*att* system is of notable value, since it has no apparent cargo-size limitation (Nuno-Gonzalez et al., 2005) and holds an increased efficiency for targeted single-site integration events (up to 60% in *Drosophila melanogaster*) due to the irreversibility of the integration reaction (Farruggio et al., 2017; Groth et al., 2004). This becomes also apparent though its successful application in diverse insect species relevant for insect biotechnology, and pest and vector control, including *Bombyx mori* (Yonemura et al., 2013), *Ceratitis capitata* (Schetelig et al., 2009b), *Anastrepha ludens* (Meza et al., 2014), *Aedes albopictus* (Labbé et al., 2010), *Aedes aegypti* (Nimmo et al., 2006), *Anopheles stephensi* (Amenya et al., 2010; Isaacs et al., 2012) and *Anopheles gambiae* (Meredith et al., 2013, 2011; Pondeville et al., 2014). In contrast, the Cre/*loxP* and Flp/*FRT* systems have only been used in *Drosophila melanogaster* for single-site transgene integration in insects (Rong and Golic, 2000). This is largely owed to low efficiencies that result from the fact that the reverse reaction (re-excision) is thermodynamically and kinetically favored over integration (Baer and Bode, 2001). Although this problem was solved for the Cre/*lox* system by the invention of the left element/right element (LE/RE)-mutant strategy – this is based

on the recombination of two *lox* sites that carry a 5 bp mutation in either the left or right inverted repeat, respectively, that after recombination create two incompatible (a LE+RE double-mutant and a wild-type) *lox* sites (Araki et al., 1997) – the Cre system did not become prevalent for site-specific single insertions in insects. This is mainly because SSR-based single-site integrations have in general the adverse characteristic of integrating the entire donor vector into the genome, including undesired prokaryotic sequences, such as the antibiotic resistance marker. This is of particular concern for bioengineered insects generated for release programs (National Research Council (US) Committee on Defining Science-Based Concerns Associated with Products of Animal Biotechnology, 2002; Wimmer, 2005b).

In order to overcome this drawback, an advanced technique named recombinase-mediated cassette exchange (RMCE) has been developed, which enables efficient and confined integration of only the desired part of a donor vector, without entraining the unwanted backbone. The rationale of RMCE is a double reciprocal recombination between two pairs of RTSs that flank both, the donor sequence as well as the genomic target sequence to be exchanged (Bethke and Sauer, 1997; Bode et al., 2000; Schlake and Bode, 1994). However, the two RTS-pairs may only be from the same SSR system, if either the system innately operates with two different RTSs that possess no self-recognition (e.g. *attP/attB* recombination of serine integrases) or if so-called heterospecific RTSs are available (e.g. *loxN*, *lox2272* or *FRT3, FRT5* for Cre or Flp, respectively). Heterospecific RTSs are slightly different variants of the same basic RTS. Although they remain substrates of the same recombinase, they cannot recombine with one another (no cross-interaction) but only amongst identical, homospecific variants (self-recognition). The variation and consequential heterospecificity is a result of functional mutations within the 8 bp spacer region. This asymmetric sequence is variable but requires full homology for recombination (Araki et al., 1997; Langer et al., 2002; Lee and Saito, 1998; Seibler and Bode, 1997).

Thus, during Cre- and Flp-RMCE only the homospecific RTSs of the genomic acceptor cassette and the cassette on the donor plasmid will recombine, leading to the substitution of the sequences flanked by the RTSs (Horn and Handler, 2005; Oberstein et al., 2005; Turan and Bode, 2011). Whilst Cre/*lox* and Flp/*FRT* system-based RMCE via heterospecific sites possess the added advantages of enabling a directional cassette exchange and allow for subsequent modifications due to the restored RTSs post recombination, these systems entail once again the drawback of having low efficiencies as soon as the donor cassette exceeds the size of the acceptor cassette (Baer and Bode, 2001; Haghighat-Khah et al., 2015). Cre- and Flp-mediated RMCE for application in insects were both initially tested in *Drosophila melanogaster* (Horn and Handler, 2005; Oberstein et al., 2005), and afterwards transferred to biotechnologically relevant insect species, including *Drosophila suzukii* (Schetelig et al., 2018), *Anastrepha suspensa* (Schetelig and Handler, 2013c), *Aedes aegypti* (Häcker et al., 2017), and *Bombyx mori* (Long et al., 2012), respectively.

RMCE approaches that employ the ΦC31-Int/*att* system (ΦC31-RMCE) do not necessarily require additional mutated RTSs variants, as recombination is only possible between the disparate *attP* and *attB* sites. *attP* and *attB* sites thus already inherently possess this feature of heterospecific spacer mutant *lox* and *FRT* sites. Yet, unlike Cre- and Flp-RMCE, the directionality of the integrated cassette in ΦC31-RMCE cannot be preassigned due to the lack of two independently acting sets of RTSs. Hence, for an RMCE-design at which the two *attP* sites in the genome and the two *attB* sites on the donor plasmid are in a head-to-head orientation, respectively, the integration-direction of the cassette is random. In another scenario, where the sets of *attP/attB* sites are oriented in a head-to-tail manner, arbitrarily either the cassette or the backbone of the donor vector will be integrated (Turan and Bode, 2011). Another drawback of current ΦC31-RMCE designs is that they do not allow for repetitive targeting of the same locus to facilitate subsequent modifications due to the irreversibility of the recombination reaction. Besides its shortcomings, ΦC31-RMCE was shown to work reliably even for very large cassettes (Venken et al., 2006) and has been successfully established in various insect species, such as *Drosophila melanogaster* (Bateman et al., 2006), *Bombyx mori* (Long et al., 2013), *Aedes aegypti* (Haghighat-Khah et al., 2015) and *Anopheles gambiae* (Hammond et al., 2016; Kyrou et al., 2018).

More recent alternative RMCE-like approaches that aspire to unite the strengths and to overcome the shortcomings of the prevailing RMCE techniques are the dual integrase cassette exchange (DICE) (Farruggio et al., 2017; Zhu et al., 2014) and integrase-recombinase mediated cassette exchange (iRMCE) (Haghighat-Khah et al., 2015). Both approaches share the same strategy to compartmentalize the two recombination reactions of RMCE by using two independent SSR systems in order to avoid unfavored pairings of the involved sites and thereby achieve a directional cassette exchange. DICE is a one-step protocol that simultaneously employs the two robust serine integrases ΦC31 and Bxb1 with their respective *att*-sites. In iRMCE, however, the cassette exchange is subdivided into a ΦC31-based integration reaction of the entire donor plasmid followed by a Cre- or Flp-mediated excision of the undesired vector backbone parts. iRMCE was devised in the vector *Aedes agypti* and the pest moth *Plutella xylostella* (Haghighat-Khah et al., 2015). Another possibility to potentially abolish these problems could be, if the recently generated functional ortholog *attP/attB* sites that carry mutations in the dinucleotide central overlap, would be implemented and used in RMCE-constructs alternately (Blanco-Redondo and Langenhan, 2018; Colloms et al., 2014; Merrick et al., 2018; Olorunniji et al., 2017).

(iii) Landing line generation and SSR-based strain-stabilization techniques

A prime advantage of SSR systems for the bioengineering of insects is their combined ability to integrate or exchange very large DNA fragments at a specific predetermined locus, which ideally has no genomic position effects that could potentially affect the expression of a transgene. However, such loci

will only be approachable for site-specific recombination upon insertion of the respective landing site(s). To this end, so-called landing strains must be generated via germline transformation using either DNA transposon-mediated random integration (2.4.1) or programmable endonuclease-based homology directed repair approaches (2.4.2.2). The latter technique is very sophisticated, requires comprehensive sequence information and necessitates knowledge about suitable genomic loci, which are prerequisites that are rarely met for non-model vector and pest organisms. With this in mind, the use of transposable elements is often still the more appropriate choice, as in this case the alleged disadvantage of integrating randomly into the genome becomes beneficial in order to screen for new suitable loci and integrate landing sites at the same time, which could be targeted after a positive evaluation of the resulting strain (Schetelig et al., 2011; Venken et al., 2009).

Nevertheless, as mentioned in chapter *2.4.1*, transposon-based genomic integrations for the generation of bioengineered insects for release programs bear the risk of being unstable when re-exposed to the corresponding or a cross-acting transposase and, therefore, require post-integration measures to ensure long time transgene stability, strain functionality, and to prevent horizontal gene transfer (Wimmer, 2003). At present there are several existing SSR system mediated strain-stabilization approaches, which can be divided into two different basic lines of action.

The first general strategy also aims for the deletion of one of the *ITRs* and, thus, shares the same rationale as the purely transposon-based approaches of Handler et al. (2004), portrayed in chapter 2.4.1. However, instead of adding an additional *ITR* and maker to the transposon donor vector, which potentially impairs the initial integration of the comprehensive transposon (including the sub-transposon), primary transposon donor vectors designed for eventual SRR system-based stabilization alternatively contain one or several RTS(s). In successfully transformed progeny, the RTS(s) can then subsequently be targeted for a recombinase-mediated integration of a construct, comprising of an additional *ITR* and a second distinct marker. This integration leads to the formation of a sub-transposon, which, depending on the exact design, includes one of the two markers. Ensuing re-exposure of the offspring that express both markers to the respective transposase, enables (besides other possible events) the mobilization and thereby, excision of the newly formed sub-transposon, leaving the transgenic construct with only one *ITR*. Flies carrying this stabilized transgenic construct can be identified, as they should only express the remaining marker, which was not part of the excised sub-transposon (Schetelig et al., 2011; Scolari et al., 2008b). This type of strategy has been realized in two variants, utilizing different SSR systems and techniques for the site-specific integration. It was first developed in *Drosophila melanogaster*, employing a Flp-RMCE with heterospecific *FRT* sites (Horn and Handler, 2005) and was later established in a modified version in the agricultural pest *Ceratitis capitata*, making use of the ΦC31-Int/*att* system to conduct a site-specific single-recombination (Schetelig et al., 2009b). Both approaches hold the added advantage that the

integration of the additional *ITR* into the inbuilt RTS(s) also opens up the opportunity to co-integrate yet another RTS and/or a transgene in tandem to the initially inserted transgenic construct, allowing the simultaneous accomplishment of strain modification and initiation of strain stabilization (Schetelig et al., 2011).

The second basic strategy does not aim for the deletion but gears towards the rearrangement of two *ITRs* between two independently inserted transposon vectors that are linked on the same chromosome (Frank Götschel, Ivana Viktorinová, Ernst A. Wimmer, unpublished). The rearrangement results from a Flp/*FRT* system-mediated recombination between two single, oppositely oriented, *FRT* sites, of which one is placed in each of the two independent transposon vectors, leading to an intra-chromosomal inversion (Golic and Golic, 1996). More precisely, the *FRT* sites are placed in both transposon vectors in the 5' UTR between a distinct promoter and the CDS of a discriminable fluorescent marker gene so that the inversion not only exchanges an *ITR* but additionally drags along the marker genes, causing a promoter-marker conversion, making the successful strain stabilization visible. This strategy was also designed in two alternative versions, deploying either two different transposase systems (e.g. *Hermes* and *piggyBac*) or the same system for the initial integration of the two transgene constructs. In the case of the former, the two transposase vectors must be oriented in opposite orientations to one another with respect to their 5' and 3' *ITR*-setups, leading, after inversion, to hybrid *ITR* combinations in both transgene constructs that are inert even in the presence of either transposase. In contrast, if both transposon vectors are being integrated using the same transposase system, their initial *ITR*-setups must have the identical relative orientation, as only then will recombination result in the generation of two transgene constructs framed by solely 5' or 3' *ITRs*, respectively, rendering the constructs refractory to the corresponding transposase (Schetelig et al., 2011).

However, these approaches require the generation and characterization of many independent lines of both transposon vectors, since they must carry the respective constructs coincidently in distinct suitable loci but on the same chromosome and in the correct orientation to one another. Only if these conditions are met will independent lines be appropriate for crossing to eventually yield a recombined line with both constructs linked on the same chromosome.

2.4.2.2 Programmable endonucleases for genome editing

Genome editing tools constitute a distinct and advanced subset of genetic engineering techniques. They comprise the unique feature to enable the site-specific modification of virtually any desired locus in the genome of an organism, including gene-insertion, -disruption, -correction, -deletion, -inversion, and chromosomal translocations (Kim and Kim, 2014). The key component of these tools are so-called "programmable endonucleases" that can be designed to bind to a certain DNA sequence where they generate a double-strand break (DSB) at an exact predetermined position (Gaj et al., 2013). The creation of the DSB is the crucial initializing step, as this activates the cellular DNA repair machinery of which the two main repair mechanisms can be exploited to edit the respective genomic locus. These two mechanisms are the error-prone non-homologous end joining (NHEJ) and the homology directed repair (HDR) pathway (Rodgers and McVey, 2016). During NHEJ both DNA ends are processed and subsequently joined together by the DNA ligase IV (Lig4) complex, which frequently results in small insertions and deletions (indels) at the cleavage site (Gorski et al., 2003). Flawless end re-ligations can occur as well, however, a restored target site is most likely only an intermediate, as it will be re-cleaved by the nuclease until an indel is eventually generated that renders the site unrecognizable to the nuclease (Chandrasegaran and Carroll, 2016). Thus, NHEJ can be exploited for targeted mutagenesis to produce, for example, gene knockouts via frameshift mutations within the coding region of a gene of interest (GOI) (Yin et al., 2017). With regard to genetic insect control this could, for instance, be a gene essential for female viability or male fertility (see 4.1; Kandul et al., 2019). Moreover, deletions or inversions as well as chromosomal translocations of up to several megabase pairs (Mbp) can be achieved through NHEJ, given that two DSBs are introduced simultaneously in *cis* or *trans*, respectively (Kim and Kim, 2014).

In contrast, HDR is capable of facilitating precise gene corrections (substitutions), deletions as well as seamless DNA insertions, which can range from single nucleotides (e.g. point mutations) to large transgene constructs of more than 17 kilobase pairs (kbp) (Gaj et al., 2016; Gantz et al., 2015). Towards this end, HDR requires a donor DNA repair template that contains the sequence to be exchanged or integrated, encompassed by additional sequences homologous to the genomic section of the DSB, termed the 5' and 3' homology arm, respectively. The repair template can be provided as plasmid (Keeler et al., 1996), or as linear ssDNA or dsDNA (Banga and Boyd, 1992; Carroll and Beumer, 2014; Nassif et al., 1994). Non-seamless and undirected insertions of exogenous DNA can also be achieved by NHEJ-mediated ligation of a linear dsDNA fragment between the two DNA ends of the DSB, which, however, can again lead to indels at the junctions (Auer et al., 2014; Maresca et al., 2013). This potential inaccuracy may render the NEHJ-approach less suitable for insertional genome editing that requires the preservation of reading frame integrity, such as in-frame gene fusions, but can be valuable for the generation of, for

example, gene-specific enhancer trap constructs, which can be integrated in less sensitive genomic loci outside of exons and gene regulatory regions (Farnworth et al., 2020; Trauner et al., 2009). Furthermore, programmable endonucleases can be utilized for the intentional destruction of one or even several entire chromosome(s), if various DSBs are generated – either by a single programmable endonuclease, given that the target sequence occurs repeatedly on the relevant chromosome (Galizi et al., 2016), or by several endonucleases that are programmed to target different sequences that are present on various chromosomes (Eckermann et al., 2014; chapters 2.3.4 & 3.2.2).

These examples, however, also demonstrate how important the availability of accurate sequence information and reliable genome annotations are in order to be able to include exact homology arm sequences to donor repair templates, and to consider fragile gene regulatory regions and exons when precisely designing unique target sequences that have no off-targets elsewhere in the genome, limiting the scope of genome editing applications to species where these requirements are fulfilled. Elaborate and frequently updated genome databases (Mohr et al., 2016; Speir et al., 2016; Thurmond et al., 2019) as well as target site selection- and off-target prediction-tools are available for all currently relevant programmable endonuclease systems for model organisms and the human genome (Bae et al., 2014; Fine et al., 2014; Gratz et al., 2014; Labun et al., 2016; Stemmer et al., 2015), but are still pending for the majority of pest and vector species.

Today, four major programmable endonuclease systems for the generation of site-specific DSBs exist, namely meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats/CRISPR-associated nucleases (CRISPR/Cas), which can be subcategorized to either protein-guided or RNA-guided programmable endonucleases, respectively, based on the kind of nuclease/DNA-interaction for target site recognition (Yin et al., 2017).

(i) Protein-guided programmable endonucleases – Meganucleases, ZFNs and TALENs

Meganucleases, also known as homing endonucleases (HE) (2.3.5), are naturally occurring, rare-cutting enzymes that possess a high level of sequence specificity conditioned by their long DNA recognition sites of commonly 12-40 base pairs (bp) (Belfort and Roberts, 1997). Several hundreds of such native nucleases have been identified in a vast variety of archaeal, bacterial, and eukaryotic microorganisms, including their associated phages and viruses (Stoddard, 2011). Depending on the array of conserved amino acids within the binding motif, HEs can be assigned to one of five different families, of which the LAGLIDADG represents the best studied family (Silva et al., 2011). Homing endonuclease genes (HEGs) frequently reside within introns that contain their cognate recognition site, which often leads to the self-induced copying of their own open reading frame (ORF) into the corresponding allele on the

homologous chromosome via cleavage induced gene conversion (Marcaida et al., 2010). This mechanism of genomic movement results in super-Mendelian inheritance, and thus enables HEGs to rapidly spread into populations, wherefore they are classified as a kind of "selfish" genetic element (2.3.5) (Beech et al., 2012; Burt and Trivers, 2008). Owing to the large recognition site, a meganuclease is likely to cleave only once, a few times or not at all within an average size eukaryotic genome. However, despite the large repertory of different HEs that provide a considerable selection of diverse recognition sites, which, furthermore, can tolerate a certain degree of sequence variability (Belfort and Bonocora, 2014; Bryk et al., 1993), the number of targetable sites is still limited and hence insufficient to target an arbitrary genomic sequence. Although noteworthy achievements have been made to engineer meganucleases for a number of specific loci (Arnould et al., 2007, 2006; Ashworth et al., 2006; Muñoz et al., 2011; Takeuchi et al., 2011), generating tailored HEs remains very cumbersome due to their overlapping DNA-binding domain (DBD) and cleavage domains (similar to common Type II restriction enzymes) (Chandrasegaran and Carroll, 2016). This only very limited programmability of meganucleases has hampered their widespread application for genome editing.

As opposed to meganucleases and common type II restriction enzymes, restriction endonucleases of the subclass type IIS, such as *FokI* from *Flavobacterium okeanokoites*, consist of two structurally and functionally distinct protein domains: a sequence-specific DBD, which recognizes a short sequence of usually < 10 bp, and a non-specific catalytic nuclease domain, that are interconnected via a polypeptide linker (Li et al., 1992; Wah et al., 1997; Williams, 2003). This modular composition made it possible to engineer chimeric endonucleases by exchanging the endogenous *Fok*I DBD for programmable DBDs with longer recognition sequences that impart higher levels of specificity to target novel and unique sites in the genome (Kim et al., 1998, 1996; Kim and Chandrasegaran, 1994). Following this concept, two platforms of targetable chimeric protein-guided endonuclease have been developed consecutively, which are the zinc-finger nucleases (ZFNs) and the transcription activator-like effector nucleases (TALENs). Thus, both, ZFNs and TALENs, contain the *Fok*I cleavage domain, but comprise different types of custom DBDs for sequence recognition linked via a five to nine, or 12 to 21 bp spacer sequence to the *Fok*I nuclease domain, respectively (Kim and Kim, 2014).

In case of ZFNs, engineered zinc finger proteins (ZFPs) are exploited for specific DNA-recognition and -binding. Such synthetic ZFPs are typically composed of an array of three to six individually selected zinc finger domains of the in eukaryotes prevalent Cys_2His_2 zinc finger motif (Gaj et al., 2013; Krishna et al., 2003), which was initially discovered in a transcription factor of the African clawed frog, *Xenopus laevis* (Miller et al., 1985). Each such domain is made up of approximately 30 aa, which corresponds to roughly 3.3 kilodaltons (kDa). Given that a single zinc finger can commonly distinguish and bind a certain nucleotide triplet, a designed ZFP is capable of recognizing a DNA sequence

of nine to 18 bp. However, the *Fok*I nuclease domain requires dimerization to obtain its DNA-cleaving activity, wherefore a second ZFN monomer needs to be designed that targets the adjacent DNA sequence on the complementary DNA strand, in due consideration of the interjacent spacer sequence in which the *FokI* domains dimerize and induce the DSB (Gaj et al., 2016). Consequently, the total target sequence length of a ZFN pair amounts to 18-36 bp (excluding the spacer sequence lying in between), which additionally enhances the specificity of ZFNs. An issue that antagonizes this enhancement is the formation of cleavage-competent homodimers of the same ZFN monomer, which again reduces the specificity and in turn evokes off-target binding, promoting cytotoxic effects (Cornu et al., 2008). In order to counteract homodimer formation different complementary heterodimeric variants of the *Fok*I nuclease domain have been devised, which, based on amino acid substitutions at the protein dimer interface, exhibit an increased affinity to heterodimerize and reduced association of domains of the same variant (Doyon et al., 2011; Gaj et al., 2016; Kim and Kim, 2014; Miller et al., 2007). While the employment of obligate heterodimers effectively minimized the level of cytotoxicity caused by ZFNs homodimers (Ramalingam et al., 2011), a more central problem regarding the modular assembly of custom zinc finger arrays to create specific ZFPs, that target new sequences, became apparent. It turned out that zinc fingers within an array frequently lose their characteristic as independent modules to recognize a defined nucleotide triplet, but often influence one another, leading to unexpected and unpredictable new DNA-binding specificities (Chandrasegaran, 2017). This is aggravated by the fact that not all the different 64 nucleotide triplets are covered by a specific zinc finger, which implies additional restrictions for target site selection. Therefore, very complicated combinatorial selection methods must be applied, which are capable of taking the changes in specificity into account that result from interactions of neighboring zing fingers within an array (Pelletier, 2016). Although several kits and online tools have been made available to ease the design and creation of custom- and self-made ZFNs (Bhakta et al., 2013; Maeder et al., 2008; Sander et al., 2011), they were shown to entail unexpectedly high failure rates, when compared to ZFNs produced by specialized companies (Kim and Kim, 2014; J.-S. Kim et al., 2010; Ramirez et al., 2008). Nevertheless, despite these difficulties ZFNs have been successfully used in a remarkable variety of species throughout the tree of life (Segal and Meckler, 2013; Urnov et al., 2010), including species relevant for insect biotechnology and vector control such as *Bombyx mori* (Takasu et al., 2010) and *Aedes aegypti*; respectively (DeGennaro et al., 2013; Liesch et al., 2013; McMeniman et al., 2014; Reegan et al., 2017).

As mentioned above, TALENs feature a related basic chimeric makeup to ZFNs. However, in this instance, the programmable DBD is comprised of transcription activator-like effectors (TALEs) motifs that originate from the plant pathogenic bacteria of the genus *Xanthomonas* (Boch and Bonas, 2010). The bacteria secrete the TALE proteins via the type III secretion system (injectisome) into the host's cells where they act as virulence factors by binding specific gene regulatory regions in the plant's genome in

order to activate expression of genes that facilitate host infection and bacterial proliferation. Such naturally occurring TALEs are composed of a central repeat DBD, flanked by additional protein segments, which encode for an N-terminal translocation signal as well as C-terminally located nuclear localization signals (NLSs) and a transcriptional activator domain (Boch et al., 2009). Large parts of the additional protein segments are dispensable for the generation of programmable DBDs for TALENs except for two cryptic repeats, termed repeat 0 and repeat -1 located upstream of the central repeat domain, plus an essential C-terminal linker sequence (Carroll, 2014; Mak et al., 2012). The central repeat domain is formed by a cluster of tandem TALE motif repeats, which constitute the building blocks for the creation of programmable DBDs for TALENs. Each repeat, apart from repeat 0 and -1, possesses almost the identical primary structure of 33-35 aa and is capable to recognize a specific single nucleotide in the DNA (Miller et al., 2011). The specificity is determined by the aa residues at the hypervariable positions 12 and 13, known as repeat variable di-residue (RVD). The most robust and predominantly used RDVs for cytosine (C), thymine (T), adenine (A) and guanine (G) are histidine-aspartate (HD), asparagine-glycine (NG), asparagine-isoleucine (NI) and asparagine-asparagine (NN), respectively (Boch et al., 2009; Moscou and Bogdanove, 2009; Pelletier, 2016). A typical TALEN monomer contains a series of 15-21 TALE repeats and the cryptic repeats that are connected via the C-terminal linker sequence (incl. NLS) to the *Fok*I nuclease domain. Therefore, TALEN pairs of two corresponding monomers, which bind DNA in opposite orientations, commonly have comprehensive target sequences of 30-42 bp in length, excluding the linker sequences (Christian et al., 2010; T. Li et al., 2011; Miller et al., 2011). With respect to the required heterodimerization of corresponding TALEN monomers in order to avoid off-target-induced cytotoxicity through homodimerization, the TALEN technology platform greatly benefited from preceding ZFNs research that had already developed the obligate heterodimerizing *Fok*I variants (see above).

As compared to other programmable endonuclease platforms, TALENs have only very minor restrictions regarding target site selection, making them particularly valuable when targeting small loci (Kim et al., 2013b). One such limitation arises from the demand for a thymine nucleotide at the position 0 at the 5' end of the target DNA sequence (5'T^0), which is recognized by the cryptic repeats (Kim and Kim, 2014; Lamb et al., 2013; Mak et al., 2012). However, protein engineering approaches amended this problem by substituting aa residues within the cryptic repeats, which yielded new TALE variants that can identify other nucleotides than the 5'T^0 (Doyle et al., 2012; Lamb et al., 2013). A second limitation becomes particularly relevant when designing TALENs for vertebrate (Bultmann et al., 2012) or plant (Kaya et al., 2017) systems. This is because the larger methylated cytosines cannot be recognized by TALE repeats holding the for regular cytosines canonical HD RVD due to steric exclusion (Jankele and Svoboda, 2014). To circumvent this issue either the thymine-specific NG RVD that is incapable of discriminating between a regular thymine and a methylated cytosine (Deng et al., 2012), or the alternate N* RVD, which

lacks an aa residue at position 13 and is therefore able to accommodate the larger methylated cytosine (Valton et al., 2012), can be employed instead. Alternatively, if applicable, chemicals that inhibit DNA methyltransferases can be utilized (Bultmann et al., 2012).

In contrast to the observed context dependent cross-interference between zinc fingers within an array, which disturbs their individual binding specificities, TALE repeats appear to have a more genuine modular property as they retain their RVD-mediated nucleotide specificities independent of neighboring repeats (Juillerat et al., 2014). Another advantage of TALEs over zinc fingers that can be attributed to the RVD-based modularity, is the simple underlying cipher between target sequence and corresponding RVD, which enables a straightforward TALE-design (Moore et al., 2014). However, while this allows a trouble-free *in silico* design of new TALENs, the actual molecular cloning of TALE arrays can become relatively laborious, as adverse recombination events may take place due to the high levels of sequence homology among the repeats (Chandrasegaran and Carroll, 2016). To remedy this downside of TALENs, several strategies to facilitate the assembly of TALE arrays have been devised, including solid-phase assembly (Briggs et al., 2012; Reyon et al., 2012; Wang et al., 2012), Golden Gate cloning based approaches (Cermak et al., 2011; Kim et al., 2013a), as well as ligation-independent cloning methods (Schmid-Burgk et al., 2013). TALENs have effectively been used for genome editing in a vast variety of viruses, organisms and cell types (Segal and Meckler, 2013; Sun and Zhao, 2013), including the vector species *Aedes aegypti* (Aryan et al., 2013; Basu et al., 2015), *Anopheles gambiae* (Smidler et al., 2013), and *Culex quinquefasciatus* (Itokawa et al., 2016), as well as in the lepidopteran model organism *Bombyx mori* (Ma et al., 2012; Takasu et al., 2016, 2013; Xu et al., 2018; Zhang et al., 2018). However, with the advent of the CRISPR/Cas technology, a new, facile genome editing platform has been devised, which has triggered an unprecedented revolution in all branches of life sciences, and consequently widely ousted the yet relatively novel TALEN technology, except for a few special applications.

(ii) RNA-guided programmable endonucleases – the CRISPR/Cas technology

RNA-guided programmable endonucleases achieve target DNA-recognition and -binding via a short non-coding RNA molecule and therefore skip labourious engineering of designed protein domains as in the case of ZFNs and TALENs. Towards this end, these monomeric endonucleases bind the RNA and form an active ribonucleoprotein (RNP) complex. Sequence complementarity of the RNA to the DNA target site guides the RNP complex to its predetermined genomic location, where the nuclease creates the DSB (Kim and Kim, 2014).

Currently, a steadily increasing number of cognate RNA-guided programmable endonuclease systems are available for genome editing that collectively originate from prokaryotic CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) adaptive immune systems

(Cebrian-Serrano and Davies, 2017), which many bacteria and the majority of archaea have evolved to identify and degrade foreign DNA of invading bacteriophages and parasitic bacteria (Barrangou et al., 2007; Bolotin et al., 2005; Mojica et al., 2000). Indeed, comparative genomics studies have disclosed that CRISPR signature genes and modules are present in almost 90% of all archaeal and approximately 50% of all bacterial genomes that have been examined so far (Grissa et al., 2007; Hille et al., 2018). However, although the constant arms race between parasite and host has led to a rapid evolution of numerous highly diverse CRISPR/Cas systems that exhibit substantial differences regarding constituents, mechanistic details, and even the type of targeted nucleic acid (Makarova et al., 2015), the general mechanism of adaptive immunity always takes place in three basic stages, namely *(1)* adaptation, *(2)* biogenesis, and *(3)* interference (van der Oost et al., 2014). During adaptation (spacer acquisition) short alien DNA sequences (~20-50 bp), also known as spacers, of the attacking viruses or bacteria are incorporated in between of similarly short invariant palindromic repeats at the CRISPR/*cas* locus, creating a CRISPR array that functions as a memory module of previously encountered invasions (Heler et al., 2014; Terns and Terns, 2011). In the course of the second stage the CRISPR repeat-spacer array is transcribed into a single continuous precursor CRISPR RNA (pre-crRNA), which is subsequently processed into short mature CRISPR RNAs (crRNAs), comprising the entire or a portion of the spacer and repeat sequences (Jiang and Marraffini, 2015). Lastly, during interference (also termed targeting stage) each crRNA forms an RNP complex with – depending on the CRISPR/Cas system – either a single multidomain Cas (CRISPR-associated) nuclease or a complex of several Cas proteins, and subsequently leads the RNP complex to its complementary sequence (protospacer) in the invading nucleic acid for cleavage (Barrangou and Marraffini, 2014; Jiang and Marraffini, 2015). Depending on whether the system employs a multisubunit Cas complex, or a large, single, multifunctional Cas nuclease for biogenesis and interference, they are assigned to either class I or class II CRISPR/Cas systems, respectively, whereby each class has additional subdivisions into several types and even more subtypes (Koonin et al., 2017; Makarova et al., 2018). However, for the sake of simplicity and convenience only class II CRISPR/Cas systems are deployed for genome editing (Jiang and Marraffini, 2015), of which the type II Cas9 nucleases are the best studied and most widely utilized RNA-guided programmable DNA endonucleases (Shmakov et al., 2017).

Cas9 proteins are so-called dual RNA-guided DNA endonucleases that are characterized by their requirement for an additional invariable, *trans*-encoded RNA, termed transactivating crRNA (tracrRNA). The tracrRNA serves as an essential cofactor for Cas9 activity, as it facilitates the binding of the crRNA to the RNP complex in the course of crRNA maturation and DNA targeting via its complementary sequence to the repeat region of the crRNA (Deltcheva et al., 2011; Jinek et al., 2012). To reduce the number of individual components necessary for genome editing applications and to circumvent the process of crRNA biogenesis, a chimeric version that links the crRNA and tracrRNA through a tetraloop to a

single guide RNA (gRNA) has been engineered (Jinek et al., 2012), which generated a simplified two-component system solely composed of Cas9 protein and gRNA. A typical gRNA is commonly designed to comprise a 20-nucleotide (nt) guide sequence at its 5', followed by 80 nt of a target-independent crRNA/tracrRNA hybrid (Carroll, 2014; Hsu et al., 2013). However, a prerequisite regarding the design of gRNAs with new guide sequences for novel target DNA sequences that must be met in order for Cas9 to create a DSB, is the presence of a protospacer adjacent motif (PAM) located on the 3' site, directly next to the target DNA sequence on the non-complementary target DNA strand (Horvath et al., 2008; Mojica et al., 2009). Recognition and binding of the PAM is mediated by the PAM-interacting (PI) domain at the C-terminus of the Cas9 protein, and is thought to prevent autoimmunity against the PAM-free CRISPR array (Hille et al., 2018; Jinek et al., 2014). PAMs are commonly two to six bp long and are highly variable among the different Cas endonucleases (Anders et al., 2014; Cebrian-Serrano and Davies, 2017). In the case of the most well-studied and predominantly utilized Cas9 from *Streptococcus pyogenes* (SpCas9) the PAM is composed of the 5'-NGG-3' trinucleotide (Deltcheva et al., 2011; Mojica et al., 2009). Once the RNP complex has assembled and found its target DNA sequence adjacent to the PAM, the guide sequence binds to the complementary DNA strand via Watson-Crick base pairing and forms an RNA-DNA heteroduplex (Jinek et al., 2014; Sternberg et al., 2015, 2014; Yang et al., 2018). This in turn displaces the non-complementary strand, which results in the local unwinding of the DNA, leading to the positioning of the complementary and non-complementary DNA strand into the respective HNH, and RuvC nuclease domain of Cas9, that together create a blunt end DSB three bp upstream of the PAM (Jiang et al., 2016; Nishimasu et al., 2014; Richardson et al., 2016).

As stated above, the prime advantage of the CRISPR/Cas technology platform for genome editing over protein-guided programmable endonucleases (2.4.2.2, *i*) is its simple, two-component design, which circumvents laborious protein engineering of DNA-binding domains. In fact, customizing the guide sequence of the gRNA is all that is necessary to route the steady Cas9 endonuclease to the target DNA at the desired genomic location (Jiang and Doudna, 2017). For this purpose, customized gRNAs can be created by either cloning-based insertion of the 20 nt guide sequence in a gRNA expression vector (Gratz et al., 2014; Port et al., 2014), or without cloning by T7-based *in vitro* transcription from a synthesized gRNA DNA template (Fu et al., 2014; Gratz et al., 2014; Kim and Kim, 2014). A potential obstacle for gRNA design that has to be taken into account, which limits the number of targetable sites, is the demand for the PAM next to the target DNA sequence (Gaj et al., 2016). However, several webtools are available that assist in finding and selecting optimal target DNA sites for guide sequence design within a given genomic sequence with due regard to the presence of a PAM (Bae et al., 2014; Hsu et al., 2013; Labun et al., 2016; Stemmer et al., 2017, 2015)

Since its emergence, the CRISPR/Cas technology has been successfully applied in an extraordinarily vast variety of organisms spanning from bacteria to humans (Sander and Joung, 2014). In insects alone, the CRISPR/Cas system has effectively been utilized in six different orders, including various pest and vectors species (Chen et al., 2016; Häcker and Schetelig, 2018; Meccariello et al., 2017; Reegan et al., 2017; Reid and O'Brochta, 2016; Sun et al., 2017; Taning et al., 2017) such as *Drosophila suzukii* (Ahmed et al., 2019; Kalajdzic and Schetelig, 2017; Li and Scott, 2016; Li and Handler, 2017) *Ceratitis capitata* (Aumann et al., 2018; Meccariello et al., 2017; Sim et al., 2019), *Bactrocera dorsalis* (Bai et al., 2019; Sim et al., 2019; Zhao et al., 2019), *Bactrocera tryoni* (Choo et al., 2018), *Anastrepha ludens* (Sim et al., 2019), *Anastrepha suspensa* (Li and Handler, 2019), *Helicoverpa armigera* (Jin et al., 2018; Wang et al., 2018; Ye et al., 2017), *Spodoptera littoralis* (Koutroumpa et al., 2016), *Spodoptera litura* (Bi et al., 2016; Zhu et al., 2016), *Locusta migratoria* (Li et al., 2016), *Tribolium castaneum* (Adrianos et al., 2018; Gilles et al., 2015; Rylee et al., 2018), *Culex quinquefasciatus* (Itokawa et al., 2016), *Aedes aegypti* (Basu et al., 2015; Dong et al., 2015; Kistler et al., 2015; M. Li et al., 2017b), *Aedes albopictus* (Park and Lyndaker, 2018), *Anopheles gambiae* (Dong et al., 2018; Galizi et al., 2016; Hammond et al., 2016; Kyrou et al., 2018), *Anopheles stephensi* (Gantz et al., 2015) and several other Malaria *Anopheles* species (M. Li et al., 2017a).

Besides its use for genome editing and other applications, such as DNA labelling and transcription modulation (Doudna and Charpentier, 2014), the CRISPR/Cas system has, furthermore, opened up the possibility to create artificial homing endonucleases genes (HEGs) that can be applied as engineered selfish genetic elements for self-sustaining genetic insect control strategies (2.3.5 & 3.3) (Burt, 2003; Gantz and Bier, 2015).

2.5 Research objectives

The overarching aim of this work was to contribute to the improvement of various aspects of transgenic insect pest control, and can be divided into the following objectives:

Objective 1: Execution of an in-depth systematic comparison and analysis of the engineered hyperactive *piggyBac* transposase variant to its wild-type version, with the aim to evaluate its behavior and efficiency for germline transformation in three different insect species – the model organism *Drosophila melanogaster,* the emerging model organism and storage pest *Tribolium castaneum*, and the invasive agricultural pest *Ceratitis capitata* (see chapter 3.1). In case of a positive assessment, this information could be particularly relevant for the development of transgenic control approaches in pest and disease vector species, in which transgenesis is poor or has not yet been successful.

Objectives 2-4: Bioengineering of "killing-sperm" transgenic male sterilization systems for the replacement of the irradiation-based reproductive sterility approach of classical SIT, and to address limitations of current transgenic SIT sterilization methods (3.2).

Objective 2: Development of a killed-sperm system in *Ceratitis capitata* as an alternative strategy to generate male sterility by provoking apoptosis in developing sperm in order to create male flies that produce unviable or no sperm (3.2.1).

Objective 3: Conceptualization of a new male sterilization system in which reproductive sterility is evoked by inducing CRISPR/Cas9-based chromosome shredding during spermatogenesis, leading to a multifactorial sterility that is similar to irradiation-based methods (3.2.2).

Objective 4: Development of a novel killer-sperm-based reproductive sterility system as proof-of-principle in *Drosophila melanogaster*, in which bioengineered males transfer deadly sperm that kills the receiving females, precluding them from re-mating, damaging fruits, or transmitting diseases (3.2.3).

Objective 5: Generation of a minimal CRISPR/Cas9-based homing sex- conversion suppression gene drive in *Drosophila melanogaster*, to evaluate the formation of cleavage resistant alleles, which could reveal the necessity for a revision of such simple gene drive designs (3.3).

3 Results

Each chapter within the results section starts with a brief description of:

- the main aim of the particular study in the context of the complete thesis,
- the authors,
- the status of the manuscript, and
- my contributions to the manuscript.

3.1 Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species

In the overall light of this thesis, this chapter presents advancements in germline transformation using a hyperactive version of the most commonly used *piggyBac* transposase. A previous study reported that the hyperactive *piggyBac* version hyPBase (iPB7) induces sterility and does not improve germline transformation efficiency in *Drosophila melanogaster* and *Aedes aegypti*. The results of our systematic analysis comparing the original versus the hyperactive (in two different codon-optimizations) *piggyBac* helper plasmids provide strong evidence that the mammalian, as well as insect codon-optimized hyperactive transposase significantly increase the efficiency of germline transformation in three different insect species from two different orders. This work corrects the previous perception and encourages scientists in basic research and applied insect biotechnology to apply this versatile tool to for the generation of transgenic insects.

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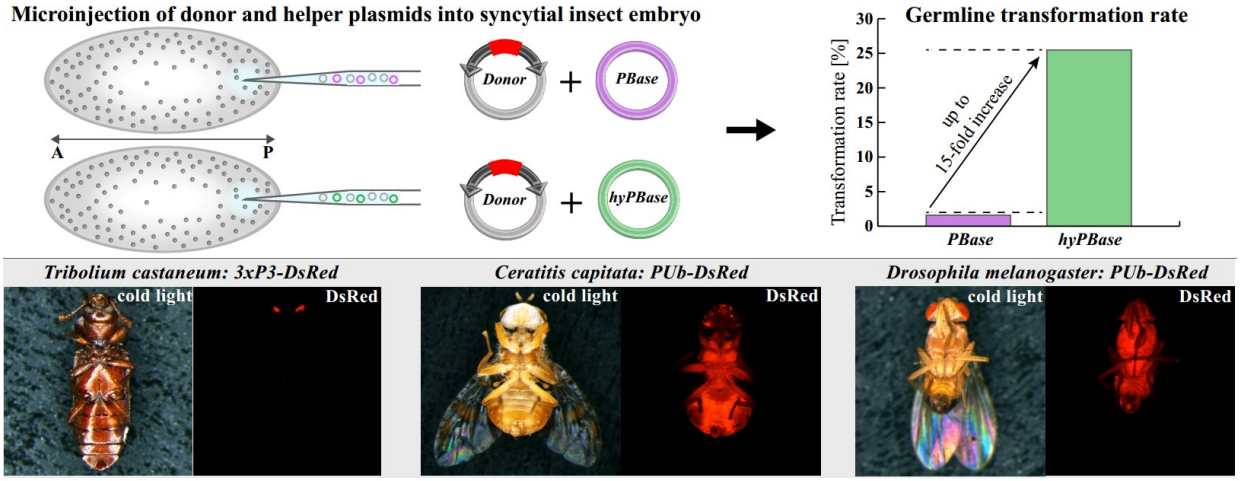
AUTHOR CONTRIBUTIONS:

As stated in the published paper:

E.A.W., **K.N.E.**, and S.D. conceived and designed the research; S.D., **K.N.E.**, and M.K. designed the helper plasmids; **K.N.E.**, M.K., P.K. and C.E.O. constructed the plasmids; **K.N.E.**, M.K., and H.M.M.A. designed the experiments and performed the injections; **K.N.E.**, M.K., H.M.M.A., and M.D.I. performed the crossings and screenings; **K.N.E.**, S.D., and H.M.M.A. performed the inverse PCR and **K.N.E.** analyzed the sequence data; **K.N.E.**, S.D., and E.A.W. wrote the manuscript; **K.N.E.** prepared the figures and tables; All authors edited and approved the final version of the manuscript.
My specific contributions were particularly to Table 3, Fig. 3 and Table 4.

Graphical Abstract



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Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species

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ABSTRACT

Even in times of advanced site-specific genome editing tools, the improvement of DNA transposases is still on high demand in the field of transgenesis: especially in emerging model systems where evaluated integrase landing sites have not yet been created and more importantly in non-model organisms such as agricultural pests and disease vectors, in which reliable sequence information and genome annotations are still pending. In fact, random insertional mutagenesis is essential to identify new genomic locations that are not influenced by position effects and thus can serve as future stable transgene integration sites. In this respect, a hyperactive version of the most widely used piggyBac transposase (PBase) has been engineered. The hyperactive version (hyPBase) is currently available with the original insect codon-based coding sequence (^{i}*hyPBase*) as well as in a mammalian codon-optimized (^{m}*hyPBase*) version. Both facilitate significantly higher rates of transposition when expressed in mammalian *in vitro* and *in vivo* systems compared to the classical PBase at similar protein levels. Here we demonstrate that the usage of helper plasmids encoding the hyPBase - irrespective of the codon-usage - also strikingly increases the rate of successful germline transformation in the Mediterranean fruit fly (Medfly) *Ceratitis capitata*, the red flour beetle *Tribolium castaneum,* and the vinegar fly *Drosophila melanogaster*. hyPBase-encoding helpers are therefore highly suitable for the generation of transgenic strains of diverse insect orders. Depending on the species, we achieved up to 15-fold higher germline transformation rates compared to PBase and generated hard to obtain transgenic *T. castaneum* strains that express constructs affecting fitness and viability. Moreover, previously reported high sterility rates supposedly caused by hyPBase (iPB7), encoded by ^{i}*hyPBase,* could not be confirmed by our study. Therefore, we value hyPBase as an effective genetic engineering tool that we highly recommend for insect transgenesis.

1. Introduction

Class II DNA transposases are enzymes that are utilized as genetic tools based on their ability to translocate DNA fragments by a "cut-and-paste-like" mechanism. The *piggyBac* transposase (PBase), isolated from a mutant Baculovirus strain in the cabbage looper moth *Trichoplusia ni* (Cary et al., 1989; Fraser et al., 1995; Handler and Harrell, 1999), is widely recognized for its broad range of targetable species and its ability to integrate large DNA cargo (Ding et al., 2005; Kahlig et al., 2010; Li et al., 2011). *piggyBac*-based elements can be excised without leaving a footprint, thus restoring the genomic locus to its pre-transposition state of the original TTAA target sequence (Elick et al., 1996).

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These features opened new possibilities for successful manipulation of various mammalian somatic and stem cell lines (Saridey et al., 2009; Wang et al., 2008; Yusa et al., 2009) and successful germline transformation in a vast variety of species including the mouse (Ding et al., 2005) as well as species of holometabolous and hemimetabolous insects (Berghammer et al., 1999; Handler et al., 1998; Nakamura et al., 2010; Tamura et al., 2000). This versatility led to its use in a wide range of scientific fields such as insect pest and disease vector control (Fu et al., 2010; Schetelig et al., 2009), gene or enhancer trap experiments (Bonin and Mann, 2004; Horn et al., 2003), the induction of pluripotent stem cells (Woltjen et al., 2009), or gene therapy (Wilson et al., 2007). Another distinguishing quality of PBase is the possibility to generate chimeric fusion constructs with e.g. DNA-binding domains to target specific genomic loci without hampering the transposition efficiency (Owens et al., 2012).

Despite the fact that there have been substantial variations in the reported relative transposition efficiencies and germline transformation rates of PBase throughout diverse target species and cell lines, direct comparisons of PBase with other transposases - including the hyperactive versions SB11 and SB12 of the previously widely used transposase Sleeping Beauty (SB) - affirmed PBase to be the most effective (Wilson et al., 2007; Wu et al., 2006). Moreover, applications in mammalian systems highly benefited from the adjustment of the codon-usage from the original - by default insect codon-based - *^{i}PBase* to a mammalian codon-optimized *^{m}PBase* version of the *piggyBac* coding sequence (CDS). This elevated the transposition efficiency up to twenty times due to increased levels of PBase protein (Cadiñanos and Bradley, 2007). Furthermore, Yusa et al. (2011) generated a hyperactive version of PBase termed hyPBase, which carries seven amino acid substitutions that were implemented into the *^{m}PBase* CDS background (*^{m}hyPBase*). Expression of *^{m}hyPBase* in mouse embryonic stem cells demonstrated an additional tenfold increase in the transposition rate when compared to *^{m}PBase* (Yusa et al., 2011). Besides, examination of *^{m}hyPBase,* and the insect codon-based equivalent *^{i}hyPBase,* confirmed the hyperactivity when compared to their wild-type counterparts for several human cell lines *in vitro* and mouse liver cells *in vivo* (Burnight et al., 2012; Doherty et al., 2012).

First results in our laboratory using *^{m}hyPBase* driven by the *Drosophila melanogaster (Dm) heat-shock protein 70* regulatory region (*Dm-hsp70*) (Lis et al., 1983) for germline transformation in the agricultural pest *Ceratitis capitata* (*Cc*) and the storage pest and emerging coleopteran model organism *Tribolium castaneum* (*Tc*), supported the literature and gave rise to approximately fourfold elevated transformation rates. This effect was even more prominent when utilizing the *T. castaneum* endogenous *heat-shock protein 68* upstream region (*Tc-hsp68*) (Schinko et al., 2012) for germline transformation in *T. castaneum* (Dippel, 2016). Consequently, hyPBase has been established in our laboratory as a standard tool. To our surprise, the publication by Wright et al. (2013) reported low transformation efficiency and high sterility rates when co-injecting the *^{i}hyPBase* helper for germline transformation in *Drosophila melanogaster* and *Aedes aegypti*.

Because of these disparate experiences to deploy hyPBase for transgenesis in insects, we decided to conduct a systematic comparison and analysis of the performance of expressed *^{i}PBase*, *^{i}hyPBase,* and *^{m}hyPBase* for germline transformation in *C. capitata*, *T. castaneum,* and *D. melanogaster*. Our data from seven large-scale injection-sets with various donor plasmids confirmed our initial observations of substantially increased transformation efficiencies and could not detect any correlations between the use of hyPBase and elevated sterility rates.

We decided to use the indices ^{i} or ^{m}, respectively, to ease the discrimination of mammalian and insect codon-optimized coding sequences of the wild-type (PBase) or hyperactive (hyPBase) transposase protein. Various nomenclatures and abbreviations have been used in the published *piggyBac* research, which are summarized in the materials and methods section.

2. Materials and methods

2.1. Nomenclature of wild-type and hyperactive piggyBac transposases and respective coding sequences

The wild-type *^{i}PBase* CDS was isolated from the cabbage looper moth and is therefore by default insect codon-based and was previously also abbreviated as *pBac* (Handler and Harrell, 1999), *PB, PBase* (Ding et al., 2005), *iPB* (Cadiñanos and Bradley, 2007), *iPBase* (Yusa et al., 2011), *pB* and *ipB* (Doherty et al., 2012).

^{m}PBase is the mammalian codon-optimized version of *^{i}PBase* and both encode the same wild-type PBase protein. *^{m}PBase* was previously also abbreviated as *mPB* (Cadiñanos and Bradley, 2007) *mpB* (Doherty et al., 2012) and *mPBase* (Yusa et al., 2009, 2011).

^{i}hyPBase stands for the insect codon-based CDS of the hyperactive transposase hyPBase and was previously also abbreviated as *i7pB, i7piggyBac* (Doherty et al., 2012) and *iPB7* (Burnight et al., 2012).

^{m}hyPBase stands for the mammalian codon-optimized CDS of the hyperactive transposase hyPBase and was previously also abbreviated as *hyPBase* (Yusa et al., 2011), *m7pB, m7piggyBac* (Doherty et al., 2012) and *hypPB* (Burnight et al., 2012). Again, both *^{i}hyPBase* and *^{m}hyPBase* encode the same hyPBase transposase.

2.2. Plasmid construction

2.2.1. Helper plasmids

The *NC-iPB7* plasmid containing the *^{i}hyPBase* CDS (Doherty et al., 2012; Burnight et al., 2012) was purchased from Transposagen Biopharmaceuticals, Inc. (Lexington, U.S.A.). Sequencing of the received plasmid revealed an undesired "eighth" mutation (V336A) that we re-mutated by site directed mutagenesis of whole plasmids as previously described (Laible and Boonrod, 2009), using the primers *iPB-7_CtoT_F* and *iPB-7_CtoT_R*, prior to further usage of the CDS for subsequent cloning steps. Primer sequences are listed in Supplementary Table 1.

The helper plasmid *Tc-^{m}hyPBase (pSL-fa_Tc-hsp_5′UTR_^{m}hyPBase_3′UTR_fa)* was generated by cloning the *^{m}hyPBase* CDS from *pCMV-hyPBase* (Yusa et al., 2011) with *KpnI/XhoI* into *pSL-fa_Tc-hsp68_5'_3′UTR_fa* (Schinko et al., 2012) between the *T. castaneum heat-shock protein 68* upstream region including the 5′ UTR and the 3′UTR. Furthermore, an undesired *NcoI*-site in the backbone of *Tc-^{m}hyPBase* was removed by *Bst*BI/*Sma*I digest, blunting with T4 DNA polymerase, and re-ligation with T4 DNA ligase. To create *Tc-^{i}hyPBase* and *Tc-^{i}PBase* corresponding transposase CDS was amplified with primers *piggyBac-NcoiFor/piggyBac-NotIrev* from templates *re-mutated_NC-iPB7* and *phsp-pBac* (Handler and Harrell, 1999), respectively, and was subsequently cloned with *Nco*I/*Not*I into *Tc-^{m}hyPBase,* replacing the *^{m}hyPBase* CDS.

The helper plasmid *Dm-^{m}hyPBase* was created by cloning the *^{m}hyPBase* CDS with *Eco*RI/*Not*I into the plasmid *pSLfaHSfa* (Ramos et al., 2006) between the upstream region plus 5′ and 3' UTR of the *D. melanogaster heat-shock protein 70*. Helper plasmids *Dm-^{i}hyPBase* and *Dm-^{i}PBase* were cloned analogously to *Tc-^{i}hyPBase* and *Tc-^{i}PBase.*

2.2.2. Donor plasmids

The donor plasmid *PK01 (pBac{3xP3-gTcv;Tc'αTub1P-Tc'H2Av-EGFP}af)* was used without further modifications (Kitzmann, 2016). To generate the donor plasmid *PK13 (pBac{3xP3-gTcv;alphaTubP-GAP43-mcherry}af)*, the chimeric CDS of the *growth associated protein-43* fused to *mCherry* was amplified via PCR from plasmid *pCS2+{GAP43-mCherry}* (a kind gift from Jubin Kashef, University Medical Center of Goettingen), using the primers *PK124_FseIGAP43Fw* and *PK122_mCherryAscIRv*. The PCR product was subsequently cloned with *Asc*I/*Fse*I into *PK01*, between the *Tc-α-tubulin1* promoter (5′) and the *SV40PolyA* site (3′).

Plasmids *pMK007* and *KNE006* were assembled with the *In-Fusion® HD Cloning System* (Takara Bio Europe/Clontech., Stain-Germain-en-Laye, France) according to the manufacturer's protocol. To create

plasmid *pMK007 (pBac{3xP3-DsRed_5xQUAS-tGFP})*, the *QUAS* and *tGFP* CDS were amplified from *pQUAST* (Potter et al., 2010) and *pSL-fa_UAS_Tc-bhsp_tGFP* (Schinko et al., 2010) with primers *QUAS_F/QUAS_R* and *Hsp68_F/tGFP_R*, respectively. The two fragments were then cloned simultaneously into the *Avr*II-linearized plasmid *#707 pBac{3xP3-DsRed > af > }* (Horn et al., 2003) via the *In-Fusion*® reaction. *pQUAST* was a gift from Liqun Luo (Addgene plasmid # 24349).

To generate plasmid *KNE006* (*pBac{attP_TREhs43-mCherry_PUbEGFP}*), the *TREhs43* and *mCherry_SV40* fragments were amplified from *KNE008 pJFRC_20xUAS_Actin5c_mCherry_SV40* and *#1402 pBac{fa_attP_TREhs43-ATGCctra-hidAla5_PUb-nls-EGFP}* (Ogaugwu et al., 2013) with primers *IF_-TRE_hs43_F/IF_TRE_ hs43_KS_R* and *IF_Cherry_SV40_F/IF_Cherry_SV40_R*, respectively. The two fragments were then cloned simultaneously into the *Asc*I-linearized plasmid *#1402* via the *In-Fusion*® reaction. To generate *KNE008* the *Actin5C* regulatory region and *mCherry* fragment were amplified from *pAC-GAL4* (Potter et al., 2010) and *pcDNA3.1/hChR2(H134R)-mCherry* (Zhang et al., 2007) with primers *IC102/IC83* and *IC86/IC91*, respectively. The two fragments were then cloned simultaneously into the *Aat*II/*Bam*HI linearized plasmid *pJFRC7-20XUAS-IVS-mCD8::GFP* (Pfeiffer et al., 2010) via the *In-Fusion*® reaction. Plasmids *pAC-GAL4, pcDNA3.1/hChR2(H134R)-mCherry* and *pJFRC7-20XUAS-IVS-mCD8::GFP* were gifts from Liqun Luo (Addgene plasmid # 24344), Karl Deisseroth (Addgene plasmid # 20938), and Gerald Rubin (Addgene plasmid # 26220), respectively.

To create plasmid *KNE007 (pXLII{Dmß2tubulin-tTA_PUb-DsRed_attP})*, the *D. melanogaster ß2-tubulin* upstream region plus 5′UTR was PCR amplified from *pCRII[ß2-tubulinP]* (Michiels et al., 1989) with primers *B2Tub-F/B2Tub-R*, which added an *Avr*II and *Xba*I cut site to the 5′ and 3′ of the amplicon, respectively. The *AvrII_Dmß2tubulin_XbaI* fragment and *#437 pXLII_attP_PUb-DsRedT3_Ccvas-tTA* (Schetelig and Handler, 2013) plasmid were restriction digested with *Avr*II/*Xba*I and subsequently ligated. *#437* was a gift from Marc Schetelig (Justus-Liebig-University Giessen, Germany).

For *KNE017 (pXLII{ACP70A-tTA; PUb-DsRed_attP})*, the *ACP70A* upstream region plus 5′UTR was amplified from genomic DNA with primers *AP70AgeIF/AP70NheIIR* which added an *Age*I and *Nhe*I cut-site to the 5′ and 3′ of the amplicon, respectively. The *AgeI_Dm-ACP70A_NheI* fragment and *KNE007* plasmid were restriction digested with *Age*I and *Nhe*I and subsequently ligated.

To generate *#1413 (pBac{Ccß2tubulin-tTA_PUb-DsRed})*, the *Ccß2tubulin-tTA* fragment was excised with *Bgl*II/*Asc*I from *#1412 pSL_Ccß2tubulin-tTA* and subsequently ligated into *Bgl*II/*Asc*I cut *#1200 pBac{fa_PUb-DsRed}* (Scolari et al., 2008). To create *#1412,* the *Ccß2tubulin* upstream region plus 5′ UTR was PCR amplified from *#1228 pSLaf_Ccß2t-tGFP-SV40_af* (Scolari et al., 2008) with primers *co109/co110,* which added an *Nco*I and *Xba*I cut-site to the 5′ and 3’ of the amplicon, respectively. The *NcoI_Cc-ß2-tubulin_XbaI* fragment was then cloned with *Nco*I/*Xba*I into the plasmid *#1225 pSLaf_srya2-tTA-SV40_af* (Schetelig, 2008).

2.2.3. DNA preparation

Helper and donor plasmids were precipitated individually. To 90 µl of an aqueous plasmid solution, containing 50 µg DNA, 10 µl 3 M NaAc and 900 µl ice-cold EtOH were added and incubated over night at −80 °C. Following centrifugation (16000 rcf, 4 °C, 30 min) the DNA pellet was washed with 70% ice-cold EtOH and again centrifuged (16000 rcf, 4 °C, 15 min), before being dried and redesolved in de-ionized H_2O. Helper and donor plasmids were mixed in a ratio of 500 ng/µl to 300 ng/µl, respectively, in 1x injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4, ph 6.8, 5% phenol red) (Bachmann and Knust, 2008) and filtered using Millex®-HV 0.45 µm (Merck Millipore, Billerica, U.S.A.).

2.3. Insect strains

Experiments in *C. capitata* were conducted with the *Egypt-II (EgII)* wild-type strain which was obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Entomology Unit, Seibersdorf, Austria). For experiments in *T. castaneum,* the white-eyed Tc-*vermillion*^{white} (*v*^{w}) strain was used (Lorenzen et al., 2002), whereas experiments regarding *D. melanogaster* were performed in the *Oregon-R* wild-type strain. All strains of the different species were maintained under their respective standard relaxed artificial rearing conditions (Brown et al., 2009; Roberts, 1998; Saul, 1982).

2.4. Germline transformation

All experiments – injection, screening, and recording of the data – were performed double-blind regarding which helper plasmid was used in the injection subsets. To reduce the impact of technical errors, which could induce additional lethality and sterility, we decided to inject only moderate volumes into the embryos. This is in contrast to experiments that do not aim for a systematic comparison but only seek for the highest possible transformation rate with the minimum effort for subsequent crossing and screening.

2.4.1. Germline transformation in Ceratitis capitata and Drosophila melanogaster

Germline transformation in *C. capitata* and *D. melanogaster* was carried out based on the principles of the previously described procedures (Bachmann and Knust, 2008; Handler et al., 1998; Spradling and Rubin, 1982) unless stated otherwise. Embryos were collected in a time interval of 30 min and were subsequently de-chorionated for 3 min in a 50% Klorix solution containing 2.5% sodium hypochlorite (DanKlorix, CP GABA GmbH, Hamburg, Germany), briefly washed in washing buffer (100 mM NaCl, 0.02% Triton X-100), washed thoroughly with double-deionized H_2O and left for 5–10 min in double-deionized H_2O to increase the internal pressure, before being fixed on double sided tape (Scotch™ Brand/3 M, St. Paul, USA) and covered with a thin layer of Voltalef 10 S oil (Lehmann & Voss & Co., Hamburg, Germany). Microinjections were performed using a FemtoJet® Microinjector (Eppendorf, Hamburg, Germany) and needles made from 10 mm × 1 mm quartz capillaries (Sutter Instrument, Novato, U.S.A.) using a P-2000 micropipette puller (Sutter Instrument, Novato, U.S.A.) applying the following settings: Heat = 750, Fil = 4, Vel = 50, Del = 125, PUL = 175. Needles were opened and sharpened using a microelectrode beveler (Bachofer GmbH, Reutlingen, Germany). All steps post embryo collection were done at 18 °C. After injection, the oil was partially drained and *D. melanogaster* embryos were kept until hatching at 25 °C and *C. capitata* embryos at 18 °C. A subset of *D. melanogaster* embryos were heat-shocked twenty hours after injection at 37 °C for 1 h. Hatched larvae were transferred into the respective larval diet at the respective temperature according to standard laboratory rearing conditions.

2.4.2. Germline transformation in Tribolium castaneum

Germline transformation in *T. castaneum* was carried out based on the principles of the previously described procedure (Berghammer et al., 1999). Embryos were collected in a time interval of 1 h and kept for one more hour at RT. The up to two-hours-old embryos were washed twice for 30 s in a 1% Klorix solution (equivalent to 0.05% sodium hypochlorite) (DanKlorix, CP GABA GmbH, Hamburg, Germany), and let dry for 5 min after aligning them into a row. Microinjection was performed using a FemtoJet® Microinjector (Eppendorf, Hamburg, Germany) and needles made from 10 mm × 1 mm borosilicate capillaries (Hilgenberg GmbH, Malsfeld, Germany) using a P-2000 micropipette puller (Sutter Instrument, Novato, U.S.A.) applying the following settings: Heat = 350, Fil = 4, Vel = 50, Del = 225, PUL = 150. Needles were opened and sharpened using a microelectrode beveler (Bachofer GmbH, Reutlingen, Germany). All steps post embryo collection were done at RT. After injection, the embryos were placed onto an apple agar plate in a sealed box at 30 °C for 72 h. Hatched larvae were transferred into vials with whole-wheat flour at 30 °C according to standard laboratory rearing conditions.

2.5. Crossings and screening

Irrespective of the species, G_0 animals were backcrossed individually to three virgin animals of the opposite sex. Crosses were monitored regularly to differentiate between sterile G_0 animals and those that have died. F_1 offspring were anesthetized with CO_2 and screened under a Leica M205 FA fluorescent stereo microscope (Leica, Wetzlar, Germany).

2.6. Inverse PCR and sequence analysis

To determine the genomic location of the *piggyBac* insertions and investigate for possible multiple insertions, inverse PCR (iPCR) was in principle carried out as described (Horn et al., 2003; Huang et al., 2000). The following modifications were introduced: genomic DNA was purified from approx. 10 adult *D. melanogaster* flies of each tested line, using the NucleoSpin® DNA Insect Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany); isolated genomic DNA was restriction digested with *Msp*I or *Mbo*I for the 5′ junction and *Bst*I or *Hind*III for the 3′ junction, respectively (Supplementary Table 2). Resulting DNA fragments were self-ligated and circularized DNA was used as template for the first PCR with primers *iPCR_5pBac_F/iPCR_5pBac_R* for the 5′ junction and primers *iPCR_3pBac_F/iPCR_3pBac_R* for the 3′ junction. An aliquot of this first PCR reaction served as direct template for subsequently conducted nested PCRs, carried out with primers *iPCR_5pBac_F_nested/iPCR_5pBac_R_nested* and primers *iPCR_3pBac_F_nested/iPCR_3pBac_R_nested*, respectively (Supplementary Table 1). Distinct DNA bands were obtained by electrophoresis and DNA was purified from cut gel slices using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The isolated DNA was Sanger-sequenced with either primer *5′-PB-SEQ* for the 5′ junction or *3′-PB-SEQ* for the 3′ junction (Supplementary Table 1). Sequences were used for BLAST search against the *D. melanogaster* genome (genome version: dmel_r6.20_FB2018_01; http://flybase.org/blast/).

3. Results

For a systematic comparative analysis of expressed *^{i}PBase*, *^{i}hyPBase* and *^{m}hyPBase* in germline transformation, we generated two independent sets of the three helper plasmids for co-injection along with *piggyBac* donor plasmids in either *C. capitata* and *D. melanogaster* or *T. castaneum*. To be able to offer an objective comparison, helper plasmids within one set are identical except for the respective transposase CDS. For embryonic injections in *Ceratitis* and *Drosophila* we used the same set of helper plasmids which all have the *Dm-hsp70* upstream region driving the expression of the transposase, whereas the *Tribolium* helper possess the endogenous *Tc-hsp68* upstream region. To enable reliable screening for transgenic F_1 offspring, all donor plasmids contained a fluorescent eye or body marker.

3.1. Performance of the hyperactive piggyBac transposase variants in C. capitata

The analysis of the germline transformation experiments conducted with the three different helper plasmids *Dm-^{i}PBase*, *Dm-^{i}hyPBase*, and *Dm-^{m}hyPBase* in combination with either of the different donor plasmids (#1413 and KNE006) in *C. capitata* (Table 1) revealed a substantially increased transformation rate (Fig. 1A) for the experiments with both helper plasmids encoding the hyPBase (12.5%–16%) compared to the wild-type PBase (0%). The codon usage of the hyperactive *piggyBac* helper plasmids had no significant influence on its performance. Furthermore, in all experiments about two-thirds (62.5%–72.4%) of the flies reaching adulthood produced offspring, with no obvious differences between the different helper plasmids (Fig. 1B, n = 228), which indicates no influence of the hyperactive *piggyBac* on the fertility rate.

Table 1
Numbers of injected embryos, survivors and fertile crosses, and their respective rates, using the helpers Dm-iPBase, Dm-ihyPBase, and Dm-mhyPBase in C. capitata.

	Helper	# Injected Embryos	# Hatched Larvae	#Pupae	# Eclosed Flies	# Dead	# Sterile	# Fertile Crosses	# Transgenic	Hatch Rate	Pupation Rate	Eclosion Rate (to Larvae)	Eclosion Rate (to Pupae)	Sterility Rate
		[Em]	[L]	[P]	[E]	[D]	[S]	[F] = E−(D + S)	[T]	= L/Em	= P/L	= E/L	= E/P	[SR] = S/(E-D)
Donor: *1413* Size: 8 kb [11.5 kb]	*Dm-^{i}PBase*	224	79	41	27	9	6	12	**0**	35.3%	51.9%	34.2%	65.9%	33.3%
	Dm-^{m}hyPBase	229	109	74	47	18	8	21	**3**	47.6%	67.9%	43.1%	63.5%	27.6%
	Dm-^{i}hyPBase	240	102	65	60	23	12	25	**4**	42.5%	63.7%	58.8%	92.3%	32.4%
Donor: *KNE006* Size: 8 kb [11.5 kb]	*Dm-^{i}PBase*	226	132	60	41	4	12	25	**0**	58.4%	45.5%	31.1%	68.3%	32.4%
	Dm-^{m}hyPBase	163	151	89	63	4	19	40	**5**	57.4%	58.9%	41.7%	70.8%	32.2%
	Dm-^{i}hyPBase	211	145	81	58	10	18	30	**4**	68.7%	55.9%	40.0%	71.6%	37.5%

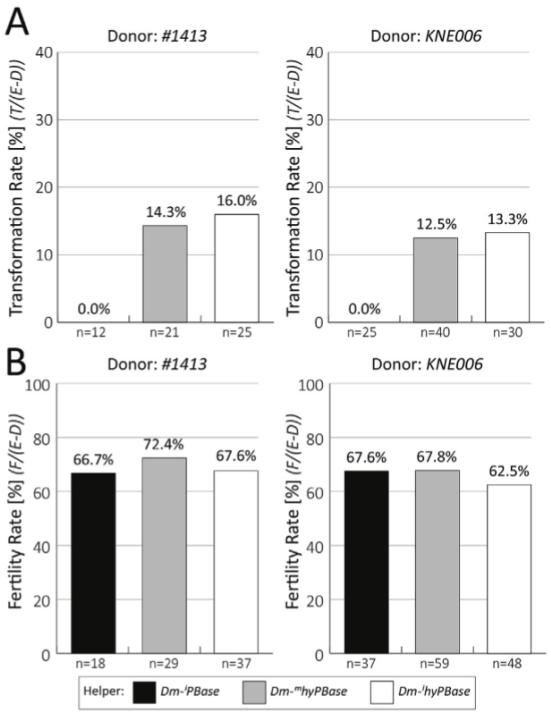

Fig. 1. Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Dm-^{i}PBase* (black), *Dm-^{i}hyPBase* (grey), and *Dm-^{m}hyPBase* (white)) in combination with two different donor plasmids (*#1413* and *KNE006*) in *C. capitata*. D, death; E, eclosed flies; F, fertile crosses; T, producing transgenic offspring).

3.2. The activity of the hyperactive piggyBac variants in T. castaneum

The systematic analysis of the germline transformation experiments in *T. castaneum* Table 2), which were performed using the helpers *Tc-^{i}PBase*, *Tc-^{i}hyPBase*, and *Tc-^{m}hyPBase* in combination with either of the three different donor plasmids (*PK13, PK01*, and *pMK007*), showed a substantial and at least doubled increase in the transformation rate by the hyPBase helpers (17.6%–39.4%) compared to the wild-type *PBase* (0%–15%), and therefore confirmed our preliminary observations as well as the results from *C. capitata*. Interestingly, we found that both hyperactive helpers remarkably improved the transformation rate of constructs *PK13* and *PK01*, which were previously difficult to transform in our lab. Also in our initial experiment, we failed to generate transgenic beetles using the wild-type helper (*Tc-^{i}PBase*). Only by repeating one injection set (Fig. 2A, *PK13*, second column) in a much larger scale, we eventually achieved a transformation rate of 1.6%. In comparison to the over 20% transformation rate obtained with both hyperactive helpers, this clearly indicates an enormous improvement and offers the possibility to circumvent problems with constructs that are difficult to transform.

In *T. castaneum* we observed in contrast to *C. capitata* a slightly better performance using the helper with the insect codon-based hyperactive transposase CDS (*Tc-^{i}hyPBase*).

Besides the much better performance of the hyPBase, we could not observe a negative effect on the survival or fertility rate in comparison to the wild-type *PBase* of the injected beetles (Fig. 2B).

3.3. The performance of the hyperactive piggyBac variants in D. melanogaster

The systematic analysis of the germline transformation experiments

Table 2
Numbers of injected embryos, survivors, fertile crosses, and their respecitve rates, using the helpers *Tc-^{i}PBase*, *Tc-^{i}hyPBase*, and *Tc-^{m}hyPBase* in *T. castaneum*.

	Helper	# Injected Embryos	# Hatched Larvae	# Pupae	# Eclosed Beetles	# Dead	# Sterile	# Fertile Crosses	# Transgenic	Hatch Rate	Pupation Rate	Eclosion Rate (to Larvae)	Eclosion Rate (to Pupae)	Sterility Rate
		[Em]	[L]	[P]	[E]	[D]	[S]	[F] = E – (D + S)	[T]	= L/Em	= P/L	= E/L	= E/P	[SR] = S/(E-D)
Donor: *PK13* Size: 5.2 kb [8.7 kb]	*Tc- ^{i}PBase*	450	144	75	75	0	3	72	**0**	32.0%	52.1%	52.1%	100%	4.0%
		2550	625	251	251	0	5	246	**4**	24.5%	40.2%	40.2%	100%	2.0%
	Tc- ^{m}hyPBase	450	94	41	41	0	0	41	**9**	20.9%	43.6%	43.6%	100%	0%
	Tc- ^{i}hyPBase	450	119	47	47	0	0	47	**12**	26.4%	39.5%	39.5%	100%	0%
Donor: *PK01* Size: 5.2 kb [8.7 kb]	*Tc- ^{i}PBase*	450	39	13	13	0	0	13	**0**	8.7%	33.3%	33.3%	100%	0%
	Tc- ^{m}hyPBase	450	35	17	17	0	0	17	**3**	7.8%	48.6%	48.6%	100%	0%
	Tc- ^{i}hyPBase	450	33	15	15	0	0	15	**5**	7.3%	45.5%	45.5%	100%	0%
Donor: *pMK007* Size: 6.6 kb [10 kb]	*Tc- ^{i}PBase*	300	36	20	20	0	0	20	**3**	12.0%	55.6%	55.6%	100%	0%
	Tc- mihyPBase	300	54	27	27	0	5	22	**8**	18.0%	50.0%	50.0%	100%	18.5%
	Tc- ihyPBase	300	76	39	39	0	6	33	**13**	25.2%	51.3%	51.3%	100%	15.4%

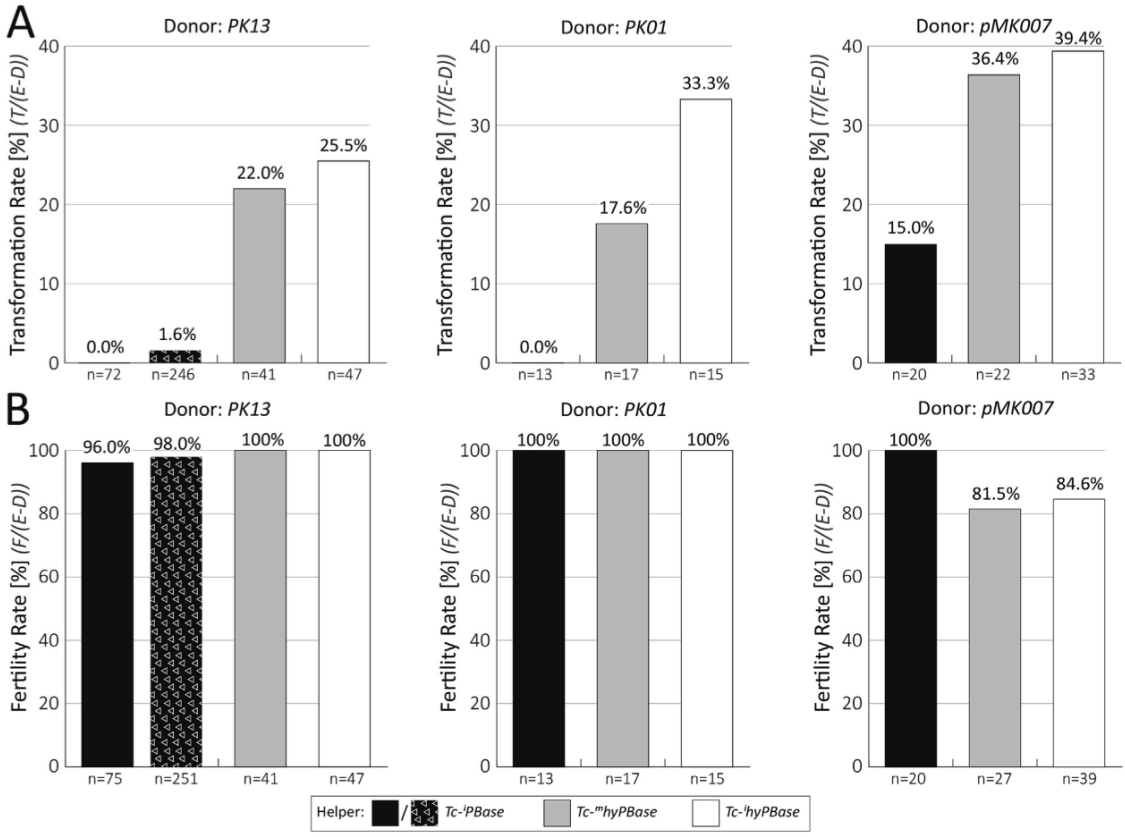

Fig. 2. Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Tc-^{i}PBase* (black), *Tc-^{i}hyPBase* (grey), and *Tc-^{m}hyPBase* (white)) in combination with three different donor plasmids *PK13*, *PK01*, and *pMK007*) in *T.castaneum*. D, death; E, eclosed beetles; F, fertile crosses; T, producing transgenic offspring).

conducted with the three different helper plasmids *Dm-^{i}PBase*, *Dm-^{i}hyPBase*, and *Dm-^{m}hyPBas* in combination with either of the two donor plasmids (*KNE007* and *KNE017*) and with or without heat-shock in *D. melanogaster* (Table 3) confirmed our previous results from the germline transformation experiments in *T. castaneum* and *C. capitata*. Under all conditions the two hyperactive helper variants outperformed the wild-type helper (Fig. 3A), with a 3–11-fold increased transformation rate compared to the wild-type helper. The codon usage of the *hyPBase* vectors had no consistent influence on the transformation rate. Heat-shock treatment however, consistently led to better performance of all tested helpers. As in the other species, also in *D. melanogaster* the fertility rate was not affected by the type of helper plasmid used (Fig. 3B).

3.4. hyPBase does not cause an increased rate of multiple insertions

During the regular *Drosophila* crossings to determine the chromosomal localization of the diverse *piggyBac* insertions, we did not find any indication for multiple insertions on different chromosomes for either of the three helper plasmids. To further investigate whether the enhanced germline transformation rate of hyPBase might result in an increased number of multiple insertion events on the same chromosome, we performed iPCR on ten lines generated by using the three different helper plasmids. In all cases, the 5′ insertion sequence matches the 3' sequence (Table 4), which argues for single insertions. Therefore, we have no implication of an increased rate of multiple insertions for hyPBase compared to PBase that already had been shown to generate rare multiple insertions (Handler and Harrell, 1999).

4. Discussion

Our results clearly show that the hyperactive *piggyBac* transposase hyPBase, regardless of the codon-usage, decidedly increases the rate of successful germline transformation compared to the wild-type *piggyBac* transposase PBase in all three tested insect species. In *C. capitata,* we were not able to produce any transgenic offspring using *Dm-^{i}PBase* at the scale of our experimental setup but reached transformation rates of up to 14.3% or 16.0% deploying *Dm-^{m}hyPBase* or *Dm-^{i}hyPBase,* respectively. In *D. melanogaster,* we achieved a 3- to 11-fold increase in germline transformation when using *Dm-^{i}hyPBase* and 5- to 8-times higher transformation efficiencies when co-injecting the *Dm-^{m}hyPBase* helper plasmid than with *Dm-^{i}*PBase. However, in *C. capitata* and *D. melanogaster* we could not observe a constant trend towards a better performance for either of the codon-usages (Figs. 1 and 3), indicating similarly effective translation of both codon-variants. Only in *T. castaneum,* the *^{i}hyPBase* helper showed consistently a slightly higher transformation efficiency than *^{m}hyPBase*, where with 39.4% (*Tc-^{i}hyPBase*) and 36.4% (*Tc-^{m}hyPBase*) also the highest transformation rates were obtained in our study (Fig. 2). Actually, our results – especially the higher transformation rates of heat-shocked compared to not heat-shocked *D. melanogaster* embryos (Fig. 3) – are consistent with the current opinion that *piggyBac* transposases lack the phenomenon of overproduction inhibition (Burnight et al., 2012; Cadiñanos and Bradley, 2007; Wilson et al., 2007).

Considering that *C. capitata*, *D. melanogaster* and *T. castaneum* belong to two different insect orders and three different families, it is reasonable to speculate that similar improvements for germline

Table 3
Numbers of injected embryos, survivors, fertile crosses, and their respective rates, using the helpers *Dm-^{i}PBase*, *Dm-^{i}hyPBase*, and *Dm-^{m}hyPBase* in *D. melanogaster* (greyscale: heat-shock).

	Helper	# Injected Embryos [Em]	# Hatched Larvae [L]	# Eclosed Flies [E]	# Dead [D]	# Sterile [S]	# Fertile Crosses [F] =E-(D+S)	# Transgenic [P]	Hatch Rate =L/Em	Eclosion Rate =P/L	Sterility Rate =S/(E-D)
Donor: *KNE007* Size: 6kb [9.5kb]	*Dm-^{i}PBase*	401	258	222	13	18	191	**6**	64.3%	86.1%	8.6%
		392	207	194	6	35	153	**8**	52.8%	93.7%	18.6%
	Dm-^{m}hyPBase	390	243	203	15	27	161	**36**	62.3%	83.5%	14.5%
		390	236	198	18	26	154	**45**	60.5%	83.9%	14.4%
	Dm-^{i}hyPBase	422	212	181	3	29	149	**15**	50.2%	85.4%	16.3%
		337	170	146	3	11	132	**31**	50.5%	85.9%	7.7%
Donor: *KNE017* Size: 6.2kb [9.7kb]	*Dm-^{i}PBase*	209	115	63	6	6	51	**0**	55.0%	54.8%	10.53%
		209	107	52	1	6	45	**1**	51.2%	48.6%	11.8%
	Dm-^{m}hyPBase	197	117	84	9	8	67	**10**	59.4%	71.8%	10.7%
		208	119	73	3	7	63	**11**	57.2%	61.3%	10.0%
	Dm-^{i}hyPBase	402	221	129	16	10	103	**18**	55.0%	58.4%	8.9%
		406	209	92	17	9	66	**17**	51.5%	44.0%	12.0%

transformation could also be achieved applying hyPBase to a variety of other insect species. This might be of particular interest when targeting non-model organisms, since their more elaborated handling during and after the injection procedure is often very labor intensive due to the species-specific biology, sensitivity to micro-manipulation and laborious artificial rearing conditions. In *C. capitata* for instance, successful

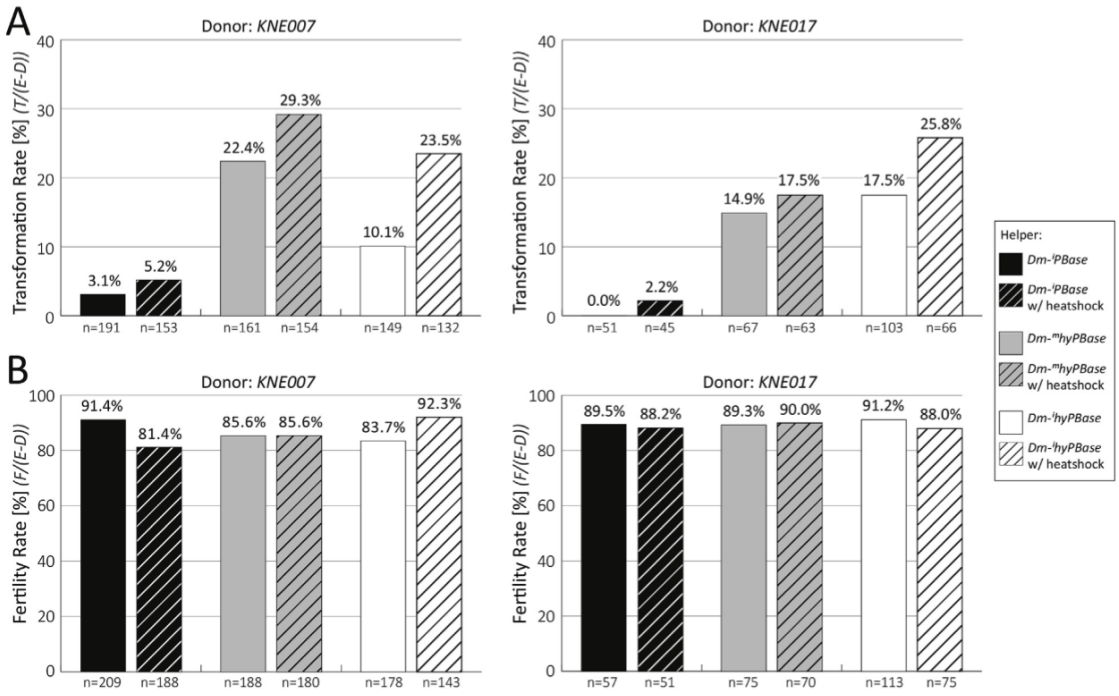

Fig. 3. Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Dm-^{i}PBase* (black), *Dm-^{i}hyPBase* (grey), and *Dm-^{m}hyPBase* (white)) in combination with two different donor plasmids *(KNE007* and *KNE017)* as well as without and with heat-shock (striped columns) in *D. melanogaster*. D, death; E, eclosed flies; F, fertile crosses; T, producing transgenic offspring).

Table 4
Insertion-site sequences isolated by inverse PCR from sets of *D. melanogaster* lines, which were generated using either of the three helper plasmids (*Dm-^{i}PBase*, *Dm-^{i}hyPBase* or *Dm-^{m}hyPBase*). In all ten examined lines only a single chromosomal insertion was detected.

Donor	Helper	Line	Genomic 5′ and 3′ Sequence of *piggyBac* Insertion	Genomic Locus of Insertion
KNE007	*Dm-^{i}PBase*	007.2	5′-tgaaaaggtaatttcacgcacttttTTAA-*piggyBac*-TTAAataacgtttcatatcgatttggttt-3′	2R:11605849
		007.4	5′-agctccgtgctaacgttgtcatcgtTTAA-*piggyBac*-TTAAtctattatttttcacccaaggtaag-3′	X:10070165
		007.7	5′-tctttaagggtgagtgacttcattgTTAA-*piggyBac*-TTAAaggctttaccacatacactctgtga-3′	3L:10473387
	Dm-^{m}hyPBase	007.3	5′-ttccagcatgctcacataattaactTTAA-*piggyBac*-TTAAataaaaacagttgtaaactatatat-3′	2R:17563360
		007.6	5′-cttgcattgcggttcaaggacatggTTAA-*piggyBac*-TTAAtggtcttccattgtgggaaagggta-3′	2R:23868999
		007.8	5′-aaatgcatgcatttcacaacaggctTTAA-*piggyBac*-TTAAagttgagcaatagatcgccagccat-3′	3L:16708553
KNE017	Dm-*^{i}hyPBase*	017.1	5′-ataggtacctgtcattcaaataacaTTAA-*piggyBac*-TTAAagctttgcaaaaaagtagctacatt-3′	2R:11639211
		017.2	5′-aataggaccgaccaccgggtattctTTAA-*piggyBac*-TTAAatattgtttacgttgcacttataac-3′	3R:9592260
		017.5	5′-atgatttaataaatatactaaataaTTAA-*piggyBac*-TTAAatatgataactgtttattgcaaaag-3′	2R:8566663
		017.7	5′-aattctcacacttcttctttcaattTTAA-*piggyBac*-TTAAaaaaggggcattgtaaaattaaaaa-3′	4:1156850

individual backcrossing is one of the most restrictive steps since their polyandrous and complex mating behavior favors mating in larger populations (Bertin et al., 2010; Bonizzoni et al., 2006), which is directly represented in the lower average fertility rate in *C. capitata* of 67.4% compared to 96% in *T. castaneum* and 88% in *D. melanogaster*.

Furthermore, cytoplasmic localization of morphogenetic determinants at the posterior pole of the developing embryo are essential for germline formation and consequently for the fertility of the adult insect. Injuries at the posterior pole can result in the loss of germ cell development, followed by a decreased percentage of fertile survivors (Swanson and Poodry, 1980). In contrast to the injection directly into the tip of posterior pole in embryos of *C. capitata* and *D. melanogaster,* the injection from the lateral site at the posterior end in *T. castaneum* embryos seems less invasive and thus poses an additional factor for the observed high average fertility rates of the adult beetles. Therefore, we assume that sterility is mainly a consequence of the injection procedure itself, the injected volume, the degree of experience of the injecting person to prevent leakage, the mating behavior of the organism and a species-specific natural variation in fertility, rather than being a direct effect of the transposase as it has been previously hypothesized for the *^{i}hyPBase* helper (Wright et al., 2013).

In ordinary germline transformation experiments in our laboratory, we would usually inject higher volumes at the expense of lower survival rates and higher sterility rates. This can be convenient as it simultaneously reduces the workload on backcrossing and screening and increases the chance that survivors of this procedure will give rise to transgenic offspring. However, to be able to accurately evaluate the possible effects of the different transposases on survival and fertility rates in this systematic comparison, we decided to not exhaust the maximally injectable volume, allowing the speculation that even higher transformation rates could be achieved.

In conclusion, the main aim of our study was to systematically test the performance of the hyperactive *piggyBac* transposase compared to its wild-type version and further elucidate whether the codon usage could make a difference for germline transformation of insects. We found that the hyperactive variant indeed functions hyperactively in all respects but could not see a general strong favor in insects for either of the insect-based or mammalian codon-optimized version. Therefore, based on our findings, we encourage molecular entomologists to consider hyPBase for future germline transformation experiments.

Author contributions

E.A.W., K.N.E., and S.D. conceived and designed the research; S.D., K.N.E., and M.K. designed the helper plasmids; K.N.E., M.K., P.K. and C.E.O. constructed the plasmids; K.N.E., M.K., and H.M.M.A. designed the experiments and performed the injections; K.N.E, M.K., H.M.M.A., and M.D.I performed the crossings and screenings; K.N.E., S.D., and H.M.M.A. performed the inverse PCR and K.N.E. analyzed the sequence data; K.N.E, S.D., and E.A.W. wrote the manuscript; K.N.E prepared the figures and tables; All authors edited and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2018.04.001.

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Supplementarty Information

Supplementary Table 1: Primers used in this study.

Primer name	*Sequence*
AP70AgeIF	*AGCATACCGGTCAGGAATAAGGTTGGCTGCTGC*
AP70NheIIR	*AGCATGCTAGCTTTTACACCGACATTCAAGCTAATCGGC*
B2Tub-F	*AATAACCTAGGACCGGTCATTGTAGGAGCCAGAGCCAATG*
B2Tub-R	*CTAATCTAGACATTTTGCTAGCAAAGTTAGGGCCCCTCTTTCAC*
co109	*ATTCGAATGGCCATGGGACG*
co110	*TTTTATCTAATCTAGACATCTTTTAAATTATCTACCGATTTAATTAC*
Hsp68_F	*CGTTTCATATATAAGCGCGGTCTCGCGGCGCGTTGTC*
IC102	*GCGGAGACTCTAGCGGAAGTACACTCTTCATGGCGATA*
IC83	*GCCTTTGCTCACCATGGTGTCTCTGGATTAGACGACT*
IC86	*ATGGTGAGCAAAGGCGAAGAAG*
IC91	*CTTCACAAAGATCCTCTAGATTATTTATACAGTTCAT CCATGCCG*
IF_Cherry_SV40_F	*TCGAATTCCAAAATGGTGAGCAAGGGCGAGGA*
IF_Cherry_SV40_R	*CGAGATCTAGGCGCGCCGGCCAGATCGATCCAGAC*
IF_TRE_ hs43_KS_R	*CATTTTGGAATTCGATATCAAGCTTATCGATGGATTG*
IF_TRE_hs43_F	*GCCGGCCTTGGCGCGCCAAG*
iPB-7_CtoT_F	*GTTATCAAAGCCTGTGCACGGTAGTTGTCG*
iPB-7_CtoT_R	*CGACAACTACCGTGCACAGGCTTTGATAAC*
piggyBac-NcoiFor	*ATATCCATGGGTAGTTCTTTAGACGATGAGCATATC*
piggyBac-NotIrev	*TCGAGCGGCCGCTCATCAGAAACAACTTTGGCACATATCA*
PK124_FseIGAP43Fw	*GTGACTGGCCGGCCATGACGTCAATGGGAGGGCAATG*
PK124_FseIGAP43Fw	*GTCAGTGGCGCGCCCTACTTGTACAGCTCGTCCATGC*
QUAS_F	*GATCGGCCGGCCTAGGCTAGCGCAAAGCTTGGCTGCATC*
QUAS_R	*CTTATATATGAAACGGCTCGAGCAATTCGATATCAAG*
tGFP_R	*GTACGGCGCGCCTAGCTTTATTCTTCACCGGCATCTG*
iPCR_5pBac_F	*ACCGCATTGACAAGCACG*
iPCR_5pBac_R	*GAACTATAACGACCGCGTGAGTC*
iPCR_3pBac_F	*GGTCGCCGACATGACACAAGG*
iPCR_3pBac_R	*CGCTTCTGACCTGGGAAAACGTG*
iPCR_5pBac_F_nested	*CTCCAAGCGGCGACTGAG*
iPCR_5pBac_R_nested	*CGTGACTTTTAAGATTTAACTCATACG*
iPCR_3pBac_F_nested	*GGTGCTTACGACCGTCAGTC*
iPCR_3pBac_R_nested	*GGTGTGTCCGTCAGTACTAGTCC*
5'-PB-SEQ	*CGACTGAGATGTCCTAAATGC*
3'-PB-SEQ	*GTTTGTTGAATTTATTATTAGTATGTAAG*

Supplementary Table 2: Restriction enzymes used for digestion of genomic DNA of the respective lines.

Line	*5' Junction*	*3' Junction*
007.2	*Msp*I	*BstY*I
007.3	*Msp*I	*BstY*I
007.4	*Msp*I	*Hind*III
007.6	*Msp*I	*BstY*I
007.7	*Msp*I	*Hind*III
007.8	*Msp*I	*BstY*I
017.1	*Mbo*I	*BstY*I
017.2	*Mbo*I	*BstY*I
017.5	*Msp*I	*Hind*III
017.7	*Msp*I	*BstY*I

3.2 Development of Killing Sperm Systems to improve the Sterile Insect Technique

A wide variety of insects can act as agricultural pests or disease vectors with devastating consequences on food production and human health worldwide. With the assistance of conventional control strategies, the Sterile Insect Technique (SIT) has proven to be a very effective, species-specific and, therefore, environmentally sound tool in area-wide integrated pest and vector management programs (Enkerlin et al., 2017; Knipling, 1960; Krafsur, 1998; Suckling et al., 2007; Vreysen et al., 2000). SIT is a "birth control" strategy, first devised in the 1940s (Klassen and Curtis, 2005; Knipling, 1955), and is based on inundative releases of sterilized males that compete with wild-type males for wild-type females to attenuate the reproductive potential of a target field population, leading to its suppression, if not eradication, from the respective area (Hendrichs et al., 2002). In classical SIT approaches male sterilization is achieved by exposing the pupae to defined dosages of gamma rays, which results in DNA fragmentation of the gonial cells that is remained in the mature sperm (Lachance, 1967; Lachance and Riemann, 1964). While this sperm is still able to fertilize eggs, such randomly introduced chromosome breaks lead to lethal chromosome aberrations (aneuploidy) in the very early embryo. Hence, irradiation-based sterilization creates multifactorial reproductive sterility, which is dissimilar from an "actual" sterility where the sperm is unviable or utterly absent. Although the random shredding of sperm chromosomes has the strength to be insensitive to resistance development, a major drawback of classical SIT is that the radiation-induced cell toxic effects are not limited to the germline but adversely affect the overall fitness of the male insects as well, impairing their mating competitiveness against wild-type males (Holbrook and Fujimoto, 1970; Hooper and Katiyar, 1971). This diminishes the overall effectiveness of classical SIT programs and can only be compensated with the release of even higher quantities of sterilized males, which, in turn, significantly increases production costs and decreases its efficacy (Parker and Mehta, 2007).

To overcome the inherent fitness problem of radiation-based sterilization, transgenic SIT approaches to induce reproductive sterility have been conceived and realized over the past two decades (Gong et al., 2005; Horn and Wimmer, 2003; Schetelig et al., 2009a; chapter 2.3.2). Regarding actual male sterility transgenic approaches, Handler (2002) postulated early on that it could be feasible to create a transgene-based system, which specifically destroys the sperm-producing cells to obtain sperm-less males (Handler, 2002a). However, insights gained from basic research on insect reproductive biology in the dipteran model organism *Drosophila melanogaster* demonstrated that sperm-transfer is essential for triggering a long-term post-mating response (PMR) in females, wherefore the concept of a sperm-less male approach was largely neglected (Kalb et al., 1993; Manning, 1967, 1962; Xue and Noll, 2000). A PMR involves a number of changes in the female reproductive behavior and physiology, including a decreased sexual receptivity to further mating and an increased oviposition rate (Chapman et al., 2003;

Chen et al., 1988; Liu and Kubli, 2003). In *D. melanogaster*, the PMR is also referred to as the "sperm-effect" and is primarily evoked by a protein termed Accessory Gland Protein 70A (ACP70A) – commonly known as Sex-Peptide (SP) – that is attached to the sperm tail, and, thus, transferred along with the sperm (Chen et al., 1988; Liu and Kubli, 2003; Peng et al., 2005; Swanson, 2003). To our knowledge, SP orthologs have only been identified in *Drosophila* species (Y.-J. Kim et al., 2010; Tsuda and Aigaki, 2016), including the fruit crop pest *Drosophila suzukii* (Ferguson et al., 2015; Schmidt et al., 1993), but seem to be absent in other dipterans of economic or medical importance, such as tephritid fruit flies or mosquitos, respectively. As it is essential that bioengineered males maintain their ability to induce PMR in order to prevent immediate re-mating of wild-type females, this might render a sperm-less male approach unsuitable for *D. suzukii*. However, recent studies in the tephritid fruit fly *Ceratitis capitata* (Gabrieli et al., 2016) and the African malaria mosquito *Anopheles gambiae* (Thailayil et al., 2011) showed that sperm-less males generated by RNAi are still capable of inducing a full PMR in females after copulation. Hence, in such species, PMR seems to be evoked solely by seminal fluid components that are transferred independent of sperm.

On the basis of these findings, we re-contemplated the idea of Handler (2002) and conceived an actual male sterility system for the major crop pest *C. capitata*, which is designed to produce transgenic male flies that have unviable, dead or no sperm present in their seminal secretion (3.2.1). For this approach, which we termed "killed-sperm" system, we combined a tissue-specific promoter/enhancer (P/E) driver element with an apoptosis inducing effector-transgene to ectopically trigger "programmed" cell death events in the sperm-forming cells.

A related transgenic approach, which also makes use of the ectopic expression of the same pro-apoptotic gene, has already been realized for the generation of a conditional male reproductive sterility system (2.3.2, *ii*). This approach was first established in our laboratory as proof-of-concept in *D. melanogaster* (Horn and Wimmer, 2003) and was transferred to *C. capitata* and *Anastrepha suspensa* thereafter (Schetelig et al., 2009a; Schetelig and Handler, 2012a). In these systems reproductive sterility is achieved through paternal inheritance of a dominant lethal transgene combination that causes early embryonic lethality (2.3.2, *ii*). However, while such systems can successfully overcome the fitness problem associated with radiation-based male sterilization and enable early female elimination, they possess a higher risk of resistance development and may be difficult to combine with transgenic embryonic sexing systems (TESSs) (2.3.2, *i*) due to their reliance on the same commonly used tetracycline repressible binary tTA expression system (TET-off system) (Gossen and Bujard, 1992; chapter 2.3.2, *i*).

To amend these issues, we conceptualized a "redundant killing" approach, which combines two independent embryonic lethality systems that are conditionalized based on two autonomous binary expression systems (Eckermann et al., 2014; chapter 3.2.2). Our proposed molecular design comprises the

existent tetracycline-repressible TESS mentioned in 2.3.2,*i*, and a new CRISPR/Cas9-based male multifactorial reproductive sterility approach, which imitates the radiation-based chromosome shredding in the sperm, and would, therefore, be more robust against resistance mutations. The shredding is to be achieved utilizing a spermatogenesis-specific promoter/enhancer to drive the expression of the Cas9 endonuclease, which will be directed by a ubiquitously expressed array of guide RNAs to cleave within conserved and frequently occurring sequences in the sperm genome. In order to be able to restrict this effect to the release generation and to allow the combined use of the two transgenic SIT approaches within one system, we furthermore suggest the application of an inducible conditional expression system that controls a site-specific recombination event necessary for Cas9 expression.

The established and novel transgenic male sterility approaches reviewed and introduced so far in this chapter aim for the release of bioengineered males that either entirely fail to fertilize eggs due to the lack of sperm or pass on an early acting dominant lethal transgene to their progeny. However, female insects commonly have a polyandrous mating behavior and can retain sperm from several males in their sperm storage organ, the spermatheca (Arnqvist and Nilsson, 2000; Scolari et al., 2014). While the transgenic embryos die during earliest development, stored sperm from matings with wild-type males can still produce viable offspring, which may considerably diminish the potency of an SIT program. Moreover, and regardless of their decreased reproductive success, female mosquitos can further on transmit diseases when imbibing a blood meal, as well as female fruit flies can continue to engage in oviposition events, which generate punctures in the fruits' skin that serve as entry points for fungal and bacterial infections, resulting again in crop yield losses (Hendrichs et al., 1995; Wimmer, 2005a).

Hence, a novel transgenic male reproductive control approach, which not only ensures sterility of the released males but also confers them with the capability to prevent females from re-mating and oviposition, or blood-feeding, respectively, could significantly enhance SIT efficiency. With this in mind, and drawing from a previously published population genetic model of a Semen-based Lethality system (SEMELE) – called after the mortal female who died after being inseminated by Zeus – (Marshall et al., 2011), we devised a "killer-sperm" system as a proof-of-principle in *D. melanogaster*, in which bioengineered males transfer deadly sperm that kills the receiving females. To this end, we want to exploit female-specific proteolytic processes that take place in the female reproductive tract post copulation, to cleave and activate a pro-toxin that is transferred with the sperm of the engineered killer-sperm males. This should then ideally lead to an induced female lethality or, at least, induce female sterility, as reproductive organs will be affected by the activated cellular toxin.

3.2.1 Development of a Killed-Sperm System in *Ceratitis capitata*

This chapter describes efforts to bioengineer a killed-sperm system in the medfly *Ceratitis capitata* as an alternative approach to induce male sterility. The system relies on the spermatogenesis-specific ectopic expression of a hyperactive version of the pro-apoptotic gene *hid* to initiate programmed cell death in developing sperm, which should result in males with dead or no sperm. The conditionality and tissue-specificity of the system are controlled by utilizing the binary TET-off expression system and the *cis*-regulatory region of the *β2t* gene, respectively. In contrast to the current transgenic reproductive sterility strategies, the killed-sperm system would provide an actual male sterility approach that could positively contribute to the ambitions to improve and broaden the repertory of transgenic sterile insect technique methods. While this study is still in progress, we here report on our advances made in cloning the *Ccβ2t-tTA* driver construct and generating *C. capitata* driver lines, as well as showing preliminary results obtained from combined lines that possess the driver and the effector construct.

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STATUS:

Work in progress

AUTHOR CONTRIBUTIONS:

- Designing research and experiments [1]
- Cloning of driver construct plasmid #1413 [2]
- Germline transformation of *C. capitata,* including microinjections, screening, and individual backcrossing of G_0 flies [3]
- Crossing driver and effector strains
- Conducting the sterility assay
- Preparing the tables and figures
- Writing the manuscript

[1] together with E.A.W.
[2] together with C.E.O.
[3] together with H.M.M.A.

Results

A repressible killed-sperm system to induce actual male sterility

The genetic design of our proposed killed-sperm system is based on the molecular principles of the embryo-specific lethality systems in *D. melanogaster* and *C. capitata* (Horn and Wimmer, 2003; Schetelig et al., 2009a). As described in 3.2 (and 2.3.2, *ii*), these two component systems contain a driver construct in which an early embryonic promoter drives the expression of the heterologous tetracycline-repressible transactivator (tTA). In the absence of tetracycline, tTA then, in turn, initiates the ectopic expression of the pro-apoptotic gene *head involution defective* (*hid*) of the TRE effector construct, resulting in death of the embryo.

hid, also known as *Wrinkled*, was first described in *D. melanogaster* and encodes a pro-apoptotic protein of the Reaper family (Grether et al., 1995). This attains its pro-apoptotic function by disrupting a complex of the anti-apoptotic Death-associated inhibitor of apoptosis 1 (Diap-1) and the initiator caspase Death regulator Nedd2-like caspase (Dronc), and thereby prevents the inhibitory ubiquitination of Dronc by Diap-1 (Goyal et al., 2000; Huh et al., 2004). This cell death inducing function of Hid, however, is abrogated when phosphorylated by the Mitogen-activated protein kinase (MAPK) of the Ras1/MAPK pathway (Bergmann et al., 1998). To evade this inhibition, the embryonic-specific lethality and sexing systems frequently utilize a phospho-acceptor residue mutant allele of the *D. melanogaster hid* (*Dmhid*^{Ala5}) gene for TRE effector construct, whose protein is insensitive to phosphorylation (Bergmann et al., 1998; Horn and Wimmer, 2003) and operative across dipteran species, including *C. capitata* (Ogaugwu et al., 2013; Schetelig et al., 2009a).

However, while we make use of the same *TRE- Dmhid*^{Ala5}-comprising *C. capitata* effector line (*TREhs43-hid*^{Ala5}_F1m2) from Schetelig et al. (2009a) (Fig. 3.2.1-1), we must direct the expression of *Dmhid*^{Ala5} to developing sperm cells in order to achieve the desired apoptosis-mediated spermicidal effect. To this end, we employed the spermatogenesis-specific promoter/enhancer element of the *C. capitata* endogenous *β2-tubulin* (*Ccβ2t*) gene (Scolari et al., 2008a) for the generation of the killed-sperm driver construct (Fig. 3.2.1-1). *β2-tubulin* is a sperm-specific isotype of the constitutive gene *β1-tubulin* and is expressed as from the start of spermatocyte development in the third larval instar (Buttgereit and Renkawitz-Pohl, 1993; Fackenthal et al., 1995, 1993; Handler, 2002a) where it plays multiple vital roles, such as in microtubule function during the following meiotic cell divisions (Fackenthal et al., 1995; Kemphues et al., 1982; White-Cooper, 2012)

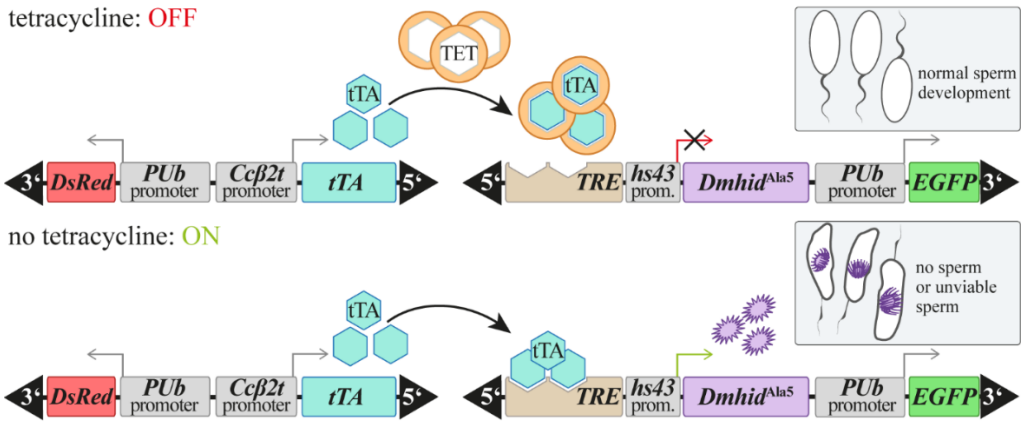

***Figure 3.2.1-1* Schematic representation of the driver/effector construct combination of the killed-sperm system and its envisaged mode of action under control of the tetracycline-repressible tTA-system.** The driver construct (left) contains a *polyubiquitin* (*PUb*) promoter/enhancer-regulated DsRed transformation marker and the *C. capitata* endogenous spermatogenesis-specific *β2-tubulin* regulatory region (*Ccβ2t*) for a targeted expression of the tetracycline-repressible transactivator (tTA) in developing sperm cells. The effector construct (right) comprises a *PUb*-regulated EGFP transformation marker and the tTA response element (*TRE*) fused to the *D. melanogaster hs43* basal heat-shock promoter that together control the expression of the phosphor-acceptor site mutant *head involution defective* allele from *D. melanogaster* (*Dmhid*^{Ala5}) (Schetelig et al., 2009a). Under permissive rearing conditions (top), i.e. in the presence of tetracycline (TET), the killed-sperm system is OFF, as TET prevents tTA from binding to the *TRE*-element, leading to an unimpeded sperm development. Under restrictive release conditions (bottom), i.e. in the absence of TET, the killed-sperm system is ON, as tTA can now effectively bind the *TRE*-element resulting in the expression of the apoptosis-inducing *Dmhid*^{Ala5} effector transgene, which should give rise to sterile males that produce no, or unviable, dead sperm.

Generation of the spermatogenesis-specific *Ccβ2t-tTA* driver construct and strains

For the generation of our killed-sperm driver construct we fused the *Ccβ2t* 5' regulatory region to the *tTA* coding sequence adjacent to a *PUb-DsRed* transformation marker (Handler and Harrell, 2001b) within a *piggyBac* donor vector to create the plasmid #1413 *pBac{Ccβ2tubulin-tTA_PUb-DsRed}* (Eckermann, 2013; Eckermann et al., 2018; chapters 3.2.4.2.2 & 3.1). This plasmid was then used for *piggyBac*-mediated germline transformation in *C. capitata*. From a total of 693 micro-injected embryos, we obtained seven independent *Ccβ2t-tTA* driver lines (chapter 3.1; Eckermann et al., 2018) that all showed a distinct expression pattern of the DsRed body-marker. Subsequently, each of the seven lines was inbred to establish homozygous driver lines of which the four lines *Ccβ2t-tTA*_DF6-P1, *Ccβ2t-tTA*_DF6-P2, *Ccβ2t-tTA* _DF14 and *Ccβ2t-tTA* _DM25 were homozygous viable, while the remaining three lines died after a few generations during the process of inbreeding.

Evaluation of actual male sterility in killed-sperm lines carrying the driver/effector construct combination

To generate the killed-sperm lines (KiSp) that carry both, the driver and effector construct, we crossed female virgins from each of the four independent homozygous *Ccβ2t-tTA* driver lines DF6-P1, DF6-P2, DF14, and DM25 to homozygous males of the *TREhs43-hid*^{Ala5}_F1m2 effector line (Schetelig et al., 2009a), to, firstly, obtain double-heterozygous flies KiSp#1, KiSp#2, KiSp#3, and KiSp#4, respectively (Fig. 3.2.1-2 A; 3.2.4.2.4). We then collected eggs from each of the four double-heterozygous

"lines" and placed them on tetracycline-supplemented food (10 µg/ml) for further inbreeding to select for homozygous flies in the next generation, as well as on a tetracycline-free diet to examine potential sterility effects already occurring in double-heterozygous fathers crossed to "con-specific" female virgins (Fig. 3.2.1-2 C; 3.2.4.2.4). However, we could not observe a reduction in the total numbers of eggs laid (data not shown), nor detect a decreased percentage in the larval hatch-rates of these eggs, from neither of the double-heterozygous KiSp flies (Fig. 3.2.1-2 C) when compared to wild-type and the homozygous driver lines (Fig. 3.2.1-2 B). As this could stem from a low dosage effect owing to the double-heterozygous state, we next equally analyzed the hatch-rates of eggs from double-homozygous parents of the four independent KiSp lines, that were cultured under restrictive conditions without tetracycline. However, similarly to the results obtained from the double-heterozygous flies, there was no noticeable difference in the hatch-rates in any of the four lines (Fig. 3.2.1-2 D). The obtained results indicate that our generated KiSp lines in their current form do not elicit the intended male sterility, which can have several explanations that will be discussed in detail in the following.

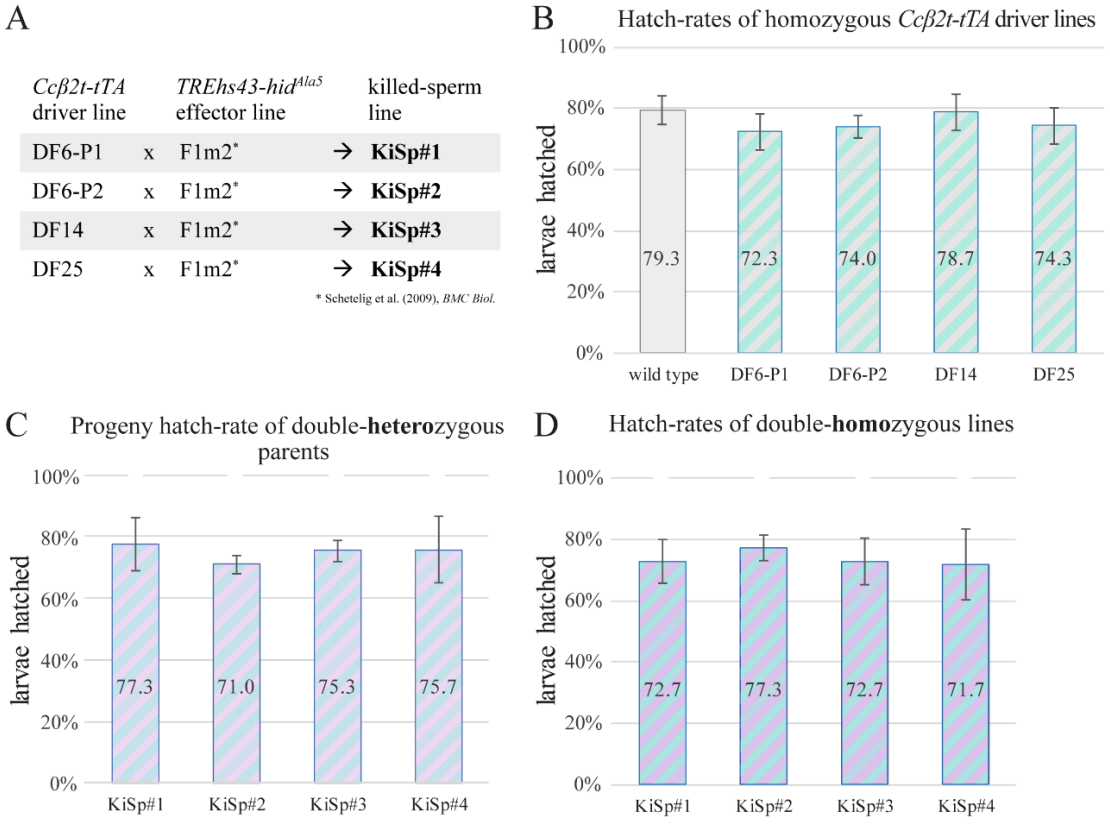

Figure 3.2.1-2 **(A) Elucidation of driver and effector line combinations for the generation of four independent killed-sperm lines. (A)** Each of the for independent homozygous *Ccβ2t-tTA* driver lines DF6-P1, DF6-P2; DF14, and DF25 were crossed separately to the homozygous *TREhs43-Dmhid*^{Ala5} effector line, which gave rise to the killed-sperm lines KiSp#1, KiSp#2, KiSp#3, and KiSp#4, respectively **(B-D) Sterility assay.** The functionality of the killed-sperm system in inducing male sterility was tested by comparing the hatch-rates of eggs from crosses where parents carried the driver/effector construct combination in (C) double-heterozygous, or (D) double-homozygous state, to hatch-rates from crosses of (B) wildtype flies and the homozygous driver lines (control)**.** The percentage of each bar is an average of the hatch-rates from three replicates of 100 embryos (n= 3 x 100 embryos). As no difference in hatch-rates could be detected, it is safe to assume that the killed-sperm system, as it is now, fails to induce apoptosis during spermatogenesis.

Discussion

In this study, we described our attempts to engineer a killed-sperm system, as an alternate transgenic male sterility approach in the insect pest *C. capitata*. To generate male flies that have no or dead sperm we aimed to induce cell death in developing sperm cells by conditional heterologous expression of the pro-apoptotic gene *Dmhid*^{Ala5} via the TET-off binary system. For this purpose, and to confine the expression to spermatogenesis, we generated a *Ccβ2t-tTA* driver construct and established four independent corresponding driver lines, which were then crossed to a pre-existing *TREhs43-Dmhid*^{Ala5} line (Schetelig et al., 2009a) to obtain KiSp lines. However, neither double-heterozygous nor double-homozygous KiSp males of the four lines showed noticeable levels of sterility when collating the hatch-rates of their progeny to those of offspring from driver and wildtype males. Since this conditional killed-sperm system consists of different components that are used outside of their endogenous context, there are a multitude of possible causes that may have led to the observed results. In order to pinpoint the source of error and find a possible solution, several common, as well as killed-sperm system specific potential pitfalls will need to be carefully reflected and investigated by a series of appropriate experiments.

One possible reason could be that neither of the four established driver lines expresses tTA, or sufficient amounts of tTA, as a result of genomic position effects that impact on the driver constructs at their site of integration (Chung et al., 1993; Wilson et al., 1990). Position effect-based silencing of transgenes can occur through nearby heterochromatin or c*is*-elements, such as silencer sequences (Kaundal et al., 2014; Wallrath and Elgin, 1995), and is a familiar problem that has already been encountered during the development of other transgenic SIT systems (Horn and Wimmer, 2003). To analyze the expression levels of tTA in our four driver lines one or a combination of several experiments should be carried out, including *in situ* hybridization in dissected testes with a probe against tTA, immunohistochemical staining of the testes with an anti-tTA antibody, and/or western blot analyses of protein extract from the testes with an anti-tTA antibody.

Another potential problem that should be considered is the combinational use of the *Ccβ2t* 5' regulatory region and the binary tTA-system in our killed-sperm approach. At least in *D. melanogaster* it was shown that employing the *Dmβ2t* 5' regulatory region to drive *Gal4* expression in the Gal4/UAS system does not lead to the expression of the *UAS*-controlled effector gene in spermatocytes (White-Cooper, 2012), for two possible reasons: (*i*) The expression peak of *β2t* is in late primary spermatocytes shortly before the transcriptional shut-down, which takes place during the two consecutive meiotic cell divisions that spawn the secondary spermatocytes and spermatids, respectively. Therefore, it is assumed that due to the lack of time, Gal4 cannot be produced in adequate amounts to initiate the expression of the *UAS*-controlled effector gene. (*ii*) This is further enhanced by a translational delay signal present in the mRNA's 5' UTR, which can be found in the 5' regulatory region of many testis-specific genes (Schäfer

et al., 1995). This mechanism ensures post-meiotic translation and, thus, supply of secondary spermatocytes and spermatids with these proteins, long after their actual peak of transcription in late primary spermatocytes. For *β2t* this translational delay is important, since it is a main constituent of the spindle apparatus of the meiotic cell divisions and the growing axoneme during spermatid elongation (Fabian and Brill, 2012; White-Cooper, 2012). While, with reference to our killed-sperm approach, this might connote that the delay signal-bearing *tTA* mRNA might indeed be translated in the spermatids after the meiotic divisions, it is rather unlikely that there is sufficient time for the produced tTA to initiate a second round of expression for the transcription of the effector construct in these cells, since post-meiotic transcription is restricted is to a minor group of genes (Barreau et al., 2008). In order to investigate this potential source of error, we will cross our driver lines to an *TREhs43-mCherry* reporter line and dissect testes from double-heterozygous F1 and double-homozygous F2 sons to check for the presence of mCherry protein using an appropriate antibody that has no cross-reactivity to the DsRed body marker of the driver lines.

Moreover, the use of a truncated *Ccβ2t*5' regulatory region, which does not contain the translational delay signal, could lead to an earlier accumulation of tTA and, thus, timely expression of the *Dmhid*^{Ala5} effector transgene. Shorter versions of the *Dmβ2t* and Cc*β2t* 5' regulatory region have already been demonstrated to induce strong and tissue-specific transcription, albeit used for direct, non-binary system-based gene expression (Michiels et al., 1989; Turkel, 2016). However, as opposed to these considerations, lately generated preliminary results in our laboratory provide indications that applying the full-length *Dmβ2t* 5' regulatory region for driving *Cas9* expression via the tTA-system in *D. suzukii* does result in detectable amounts of Cas9 mRNA by *in situ* hybridization, despite of the potential translation delay signal (Ahmed, 2019). Whether this is specific to *D. suzukii* or not is not yet clear. Therefore, it might be worthwhile to contemplate 5' regulatory regions of other spermatogenesis-specific genes for utilization in our killed-sperm driver construct that are expressed during earlier stages of spermatogenesis, such as the spermatogonia-specifically expressed gene *bag-of-marbles* (*bam*) (McKearin and Spradling, 1990). An earlier expression of *tTA* using the *bam* 5' regulatory could consequently ensure enough time for tTA to activate the expression of *Dmhid*^{Ala5} before the transcriptional shut-down.

The *TREhs43-Dmhid*^{Ala5} effector line used in this study has been generated in 2009 (Schetelig et al., 2009a). Therefore, sequencing of the genomic locus of the construct could exclude that mutations have occurred during the years of passage, which may have rendered the line nonfunctional. Furthermore, the effector line could be re-examined for functionality in the original system. However, despite the fact that this effector line has been shown to function efficiently during embryogenesis in the original system, which is facilitated by accessible chromatin at the site of integration, we cannot exclude chromatin inaccessibility during spermatogenesis that might prevent the expression of *Dmhid*^{Ala5}. The usage of

insulator sequences 5' and 3' of the effector construct, such as the chicken *β-globulin* 5' HS4 or *gypsy* transposon insulator element, could help to amend this issue (Sarkar et al., 2006).

Another aspect regarding the effector, which should be considered is that Hid has an important non-apoptotic function during spermatogenesis and might therefore be subject to an even stricter regulation in this tissue (Huh et al., 2004). This may consequently imply that Hid is not suitable for the ectopic induction of apoptosis in this context, wherefore the application of other effector transgene candidates should be considered as well.

In a recently published study by Yamamoto and colleagues, an approach very similar to our conditional killed-sperm system was developed in the Asian malaria vector *Anopheles stephensi* (Yamamoto et al., 2019). In this approach, male sterility was induced by employing the *An. stephensi* endogenous *β2t* 5' regulatory region (*Asβ2t*) to express the pro-apoptotic murine *B-cell leukaemia/lymphoma 2-associated X protein* (*mBax*) gene. In mammalians, the pro-apoptotic property of Bax is based on its role in permeabilizing the outer mitochondrial membrane, which leads to the release of Cytochrome C and the assembly of the apoptosome (Galindo et al., 2009; Gaumer et al., 2000). Males carrying the *Asβ2t-mBax* construct show strong apoptosis-induced cell death in spermatocytes, which results in the development of aberrant testes and a complete absence of sperm. Moreover, the authors provided data showing that the viability and competitiveness of these males are not compromised and that they maintain their ability to induce a full PMR in females after copulation. Although this study convincingly demonstrates the potential of *mBax* as an effector transgene to trigger apoptosis in the testes of a dipteran species, the system will still require substantial modification for conditionalization if it is to be considered for application in vector control. Therefore, and in view of the above-mentioned possible obstacles, it will be interesting to see if the system can remain to be functional when expressed via a binary expression system.

Material and Methods

For materials and methods please view section 3.2.4.

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3.2.2 Perspective on the combined use of an independent transgenic sexing and a multi-factorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches

In classical SIT, reproductive male sterility is achieved through the application of ionizing radiation that leads to the formation of sperm with fragmented chromosomes, which ultimately causes early embryonic lethality in consequence of genetic imbalance. While this has the core advantage to create a multifactorial sterility that is unsusceptible to resistance development, irradiation also affects male fitness and competitiveness. To overcome this shortcoming, we propose a novel CRISPR/Cas9-based sterility system in this chapter, that mimics the effect of ionizing radiation by inducing spermatogenesis-specific chromosome shredding, however, without imposing detrimental health effects on males. Furthermore, our transgenic design addresses another issue that is present in current transgenic SIT approaches. Most, if not all, of these approaches are conditionalized by the same TET-off binary system, which impedes their combined use and increases the probability of a genetic breakdown of the system. We, therefore, conceptualized the application of the Q-system, in conjunction with a site-specific recombinase, as a second and independent diet controllable binary expression system, which enables combining the TET-off-based sexing system with our proposed novel sterility approach to establish "redundant killing".

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Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches

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Abstract

Background: The Sterile Insect Technique (SIT) is an accepted species-specific genetic control approach that acts as an insect birth control measure, which can be improved by biotechnological engineering to facilitate its use and widen its applicability. First transgenic insects carrying a single killing system have already been released in small scale trials. However, to evade resistance development to such transgenic approaches, completely independent ways of transgenic killing should be established and combined.

Perspective: Most established transgenic sexing and reproductive sterility systems are based on the binary tTA expression system that can be suppressed by adding tetracycline to the food. However, to create 'redundant killing' an additional independent conditional expression system is required. Here we present a perspective on the use of a second food-controllable binary expression system - the inducible Q system - that could be used in combination with site-specific recombinases to generate independent transgenic killing systems. We propose the combination of an already established transgenic embryonic sexing system to meet the SIT requirement of male-only releases based on the repressible tTA system together with a redundant male-specific reproductive sterility system, which is activated by Q-system controlled site-specific recombination and is based on a spermatogenesis-specifically expressed endonuclease acting on several species-specific target sites leading to chromosome shredding.

Conclusion: A combination of a completely independent transgenic sexing and a redundant reproductive male sterility system, which do not share any active components and mediate the induced lethality by completely independent processes, would meet the 'redundant killing' criteria for suppression of resistance development and could therefore be employed in large scale long-term suppression programs using biotechnologically enhanced SIT.

Background

Many insects heavily damage agriculture and forestry or transmit deadly diseases to animals and humans. Current control efforts still mostly rely on the use of insecticides, but chemical control is not always harmless and the costs of developing new chemical compounds to overcome the world-wide threat of insecticide resistance are escalating [1]. Moreover, to protect biodiversity the establishment of pest-specific management methods is desirable. The Sterile Insect Technique (SIT) is a species-specific genetic control approach that acts as an insect birth control measure, which relies on the mass rearing, sterilization and field release of large numbers of insects. The competition between released sterile and resident males for mating with wild females leads to the reduction of the reproductive potential. If continued releases of

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high-quality sterile males in inundating numbers over several consecutive generations are performed, a progressive reduction of the population size and eventually the total eradication of the pest population will occur [2,3]. SIT is now an accepted component of various integrated approaches to control, suppress, prevent, or even eradicate invasive insect pest species from islands, large fruit production areas, or even complete continents [4]. Classically, both male and female insects were released, particularly because the distinction between male and female pupae is hardly manageable or requires the development of genetic sexing strains [5]. Released females, however, although sterile, sting fruits with their ovipositors or keep blood feeding and potentially transmit diseases as well as compete against wild females for mating with the sterile males [5]. In addition, sterilization is classically achieved by irradiation, a procedure that often renders insects very weak and unfit to compete with the wild mates [6]. Such drawbacks and many years of experience have put forward several key requirements for an efficient SIT application: intensive rearing of large numbers of insects for mass release, the availability of efficient sex-separation methods, sterilization techniques able to handle large numbers of insects with minimal effects on fitness and competitiveness, effective release methods, and efficient marking systems to identify released individuals during monitoring of SIT programs.

Biotechnological engineering of insects makes novel approaches possible to efficiently mark insects as well as selectively produce vigorous and potent sterile males, which are generated by conditional male reproductive sterility in combination with conditional female lethality. This will improve efficacy and widen applicability to further insect pest species [7,8]. To minimize the concerns coupled with the release of transgenic organisms, SIT programs are actually ideal, as the sterility of the released males will serve as a biological safety mechanism for containment as it impedes the spread of transgenes and allows for a safe deployment [9,10].

In accordance to this hope for novel successful genetic pest management strategies, the first biotechnologically engineered designer insects have already been released in small scale trials: pink bollworm moths in Arizona, USA [11], as well as yellow fever mosquitoes in the Grand Cayman Islands [12], Malaysia [13], with a currently ongoing release in Brazil [14,15]. For the release in the Grand Cayman Islands, it has been shown that the sustained release of transgenic mosquitos carrying a dominant lethal gene could successfully suppress a field population [16] demonstrating the great potential of transgenic SIT approaches. Envisioning the beneficial future use of genetically modified insects, the European Food Safety Authority has recently published a scientific opinion on the guidance on the environmental risk assessment of genetically modified animals including insects [17]. Since reproductive sterility based on lethality systems serves as an intrinsic containment against vertical transmission of transgenes in biotechnologically engineered SIT, its application does not present real concerns in respect to humans and the environment [18].

Nonetheless, the use of transgenic SIT approaches is still at initial stages and an ongoing large scale use somewhat premature, as potential resistance development might pose a significant threat to the further use of this technology [19]. In the currently released transgenic mosquitoes, the dominant lethality is mediated by the overexpression of a synthetic transcription factor that is deleterious to cells at very high levels reached by auto-activation in a positive feedback loop [20]. This presents just one single killing system based on an unclear mechanism. Since most pest insects produce large numbers of offspring, they have a high propensity to evolve resistance to control measures. Actually classic SIT based on sterilization by irradiation is an exception in the resistance development context, as the radiation-induced breaks of the chromosomes are random and vary among all individuals thus providing built-in redundancy [21]. However, transgenic SIT approaches with defined killing systems are in principle susceptible to resistance development. Thereby, we assume that the released insects still contain functional transgenes and are themselves susceptible to the dominant lethality [22]. The potential break down of transgenic characters during mass rearing is an additional important but different issue for quality control before release. In respect to resistance development the heterogeneous genomes of the field populations are important [21], which might contain genotypes that lead to suppression or partial suppression of the lethality traits. For the avoidance of behavioural resistance, where wild type insects reject mass-reared insects as mating partners, regular introgression of wild type genetic material into the mass rearing strains has been successful [3]. However, there is also the possibility of biochemical resistance to biotechnologically engineered lethality. Due to the inundation of the population with susceptible alleles by the release of the sterile insects during an ongoing SIT program, only strong resistance-mediating alleles acting dominant and having only low fitness costs propose a threat to SIT programs but are so far only hypothetical [22].

Nevertheless, insects have successfully developed resistance to synthetic chemicals as well as to microbial agents [23] and are also likely to develop resistance to transgenic SIT approaches when employed in long-term suppression programs [24]. One strategy to significantly impede or at least delay resistance development could be based on the principle of 'redundant killing' [25,26]. Therefore, transgenic SIT strains with effective and

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necessary sterility or lethality traits should only be considered in large scale long-term suppression programs, once completely independent toxicity systems have been combined. Since actually two traits are favourably introduced by transgenesis - female lethality for male only releases as well as reproductive sterility by dominant lethal transgenes - one task is to identify two completely independent ways of mediating them.

Combination of two independent systems: male reproductive sterility and female lethality

A sterile insect in the sense of SIT is defined as "an insect that, as a result of a specific treatment, is unable to reproduce" [27]. A first approach to cause such reproductive sterility by biotechnological engineering was successfully demonstrated in the non-pest insect *D. melanogaster* [28]. The system is based on the transmission of a transgene combination that causes conditional embryo-specific lethality in the progeny without larval hatching and has successfully been transferred to tephritid fruit flies [29,30]. This prevents larval damage to fruits and the introgression of transgenes into wild type fruit fly populations. Furthermore, for tephritid fruit flies and mosquitoes, transgenic strains were produced using an autocidal overexpression loop of the protein tTA, which leads to dominant lethality when transgenic males were mated to wild type females [20,31]. Additional transgenic reproductive sterility systems [32,33] might be based on species-specific homing endonucleases [34].

To generate transgenic sexing systems, female lethality was first developed and tested in *D. melanogaster* and based on the female-specific expression of conditional lethal genes [35,36]. More recently transgenic sexing systems for tephritid fruit flies have been generated using a female-specifically spliced intron from the *transformer* gene. First it was used in an autocidal expression loop with the female lethality occurring at late larval stages in the Medfly *Ceratitis capitata* [37]. This system has successfully been transferred to other Tephritids such as the olive fly *Bactrocera oleae* [38] and also to blowflies [39]-devastating pests of livestock - as well as to lepidopterans [40]. Furthermore, embryonic transgenic sexing systems have combined the use of such a female-specifically spliced intron with an early embryonic expression mediated by *cis*-regulatory elements from early acting cellularization genes that indirectly and controllably drive the expression of a hyper-active pro-apoptotic gene (Figure 1) [41,42]. An even better understanding of the sex differentiation pathways in insects will provide us with additional strategies for synthetic genetic-based tools for large scale sex separation in SIT applications [43] based on either female killing or actual female sex-reversal [44,45].

tTA: the commonly used conditionally repressible expression system

The conditionality of the so far established transgenic sexing and reproductive sterility systems is based on a binary expression system, which can be suppressed by supplementing the food with tetracycline (Figure 1). The tetracycline-controlled transactivator (tTA) consists of a bacterial-viral fusion protein [46] that activates gene expression after binding to a tTA-response element (*TRE*). The major advantage of this binary expression system is that a food supplement can suppress the activation providing an additional control to the directed gene expression [47]. tTA complexed with tetracycline is prevented from binding to its response element and the downstream gene is not activated. The expression system is thus switched off by supplementing the food with tetracycline, which allows for an additional control on top of the tissue-specific promoter driving tTA expression. Since only small amounts of tetracycline are needed to suppress the expression, this system has become the most favourable expression system to develop transgenic SIT approaches. However, to create a situation of 'redundant killing' based on two completely independent mechanisms to mediate reproductive sterility and female lethality, an additional conditional expression system is necessary.

Second food-controllable expression system: Q system

Recently a second food-additive controllable expression system - the Q system - has been shown to work *ex vivo* in mammalian cells as well as *in vivo* in the vinegar fly *D. melanogaster* [48,49]. The broad applicability of this system is also demonstrated by its functionality in the nematode worm *Caenorhabditis elegans* [50]. The Q system is based on the regulatory genes of the gene cluster *qa* from the bread mold *Neurospora crassa*, which allows the fungus to utilize quinic acid as a carbon source [51]. Quinic acid can be found in high concentrations both in herbaceous plants as well as conifers [52] and at especially high levels in unripe fruits [53]. Several molds are able to use quinic acid as carbon source and have specific gene clusters for the catabolic pathway [54]. The regulatory genes of the cluster ensure that the catabolic enzymes are only expressed at the presence of quinic acid: one gene, *qa-1F* (*QF*), acts as DNA-binding transcriptional activator of all cluster genes, whereas another regulatory gene, *qa-1S* (QS), acts as a repressor that does not bind DNA itself but inactivates the activator QF by complex formation [54]. Quinic acid acts as an inducer by hindering the repressor QS from complexing QF, which then can activate its target genes (Figure 2). Therefore, the Q system is actually an inducible binary expression system with the food additive, quinic acid, leading to the activation of controlled gene expression. This and the fact that quinic acid

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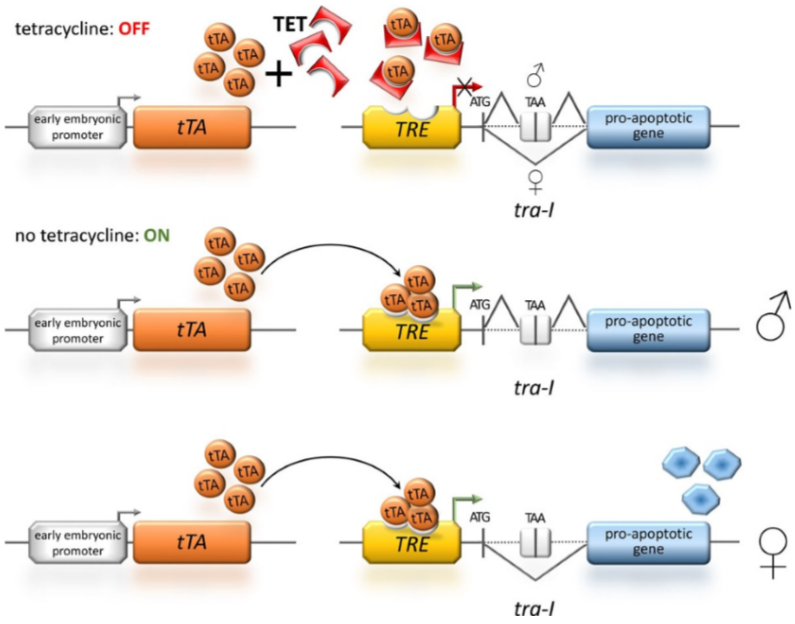

Figure 1 Sexing using female-specific splicing under the control of the repressible tTA-system. The depicted transgenic sexing system [41,42] uses a sex-specifically spliced intron and a hyperactive pro-apoptotic gene to generate female-specific lethality under the regulation of the tetracycline-controlled transactivator (tTA). To cause early embryonic lethality and thus avoidance of larval survival, the tTA is under the control of an early embryonic promoter. During rearing of such strains, addition of tetracycline (TET) to the food keeps the system in the OFF state, as tetracycline blocks the binding of tTA to its response element (*TRE*). For the release generation, tetracycline is absent in the food and therefore the sexing system is ON: in males, the male specific splicing of the transformer intron (*tra-I*) - placed after the translation start codon (ATG) of the effector gene - includes a small exon containing a TAA stop codon between the start codon and the rest of the effector gene and therefore prevents the production of the functional pro-apoptotic effector protein allowing the males to survive; whereas in the females the female specific splicing of the *tra-I* produces a functional effector and the embryonic cells are driven into apoptosis, which leads to female-specific embryonic lethality.

is found widespread in nature [52,53] do not allow us to use this system in an analogous way to the tTA system. However, it offers a completely independent food additive-controlled expression system that should be utilized for novel transgenic SIT approaches.

Render inducible system suitable for transgenic SIT approaches

An inducible system would usually require that the inducer is constantly present to have the system activated. But as this cannot be warranted for a food-additive after release, a temporary induction of the system needs to be stabilized into a continuous expression. For this purpose site-specific recombination systems [55] can be utilized to stabilize an inducer pulse into a persistent activation. For the *flp* recombinase (FLP), it was demonstrated in *D. melanogaster* that a region-specific promoter can be separated from the downstream coding region by a *flp*-out cassette that contains a transcriptional terminator and is flanked by *flp* recombinant target sites (FRTs) [56,57]. The transcriptional terminator prohibits the directed expression mediated by the tissue-specific promoter until FLP removes the *flp*-out cassette by site-specific recombination of the FRTs that are in direct orientation (Figure 2). The left over single FRT in the 5'UTR does not interfere with effective transcription and translation of the downstream coding sequences [56,57]. On this basis, the Q binary system can be combined with the FLP mediated transcriptional activation system to stably activate the expression of a gene after a pulse induction with an inducer (Figure 2).

To reduce the number of constructs necessary for such a complex inducible Q and immediate targeted gene expression system, actually the regulatory components of the Q system can be placed into the *flp*-out cassette (Figure 2) which will also place the Q system components under the same control as the later expressed effector gene [57]. To actually place both regulator genes - QF and QS - into the same construct, the two coding regions can be separated by an internal ribosome entry site (IRES) to allow for a bi-cistronic transcript. Depending on the translational start efficiency of the insect virus IRES compared to the actual capped mRNA [58], the QS and QF coding sequences should be

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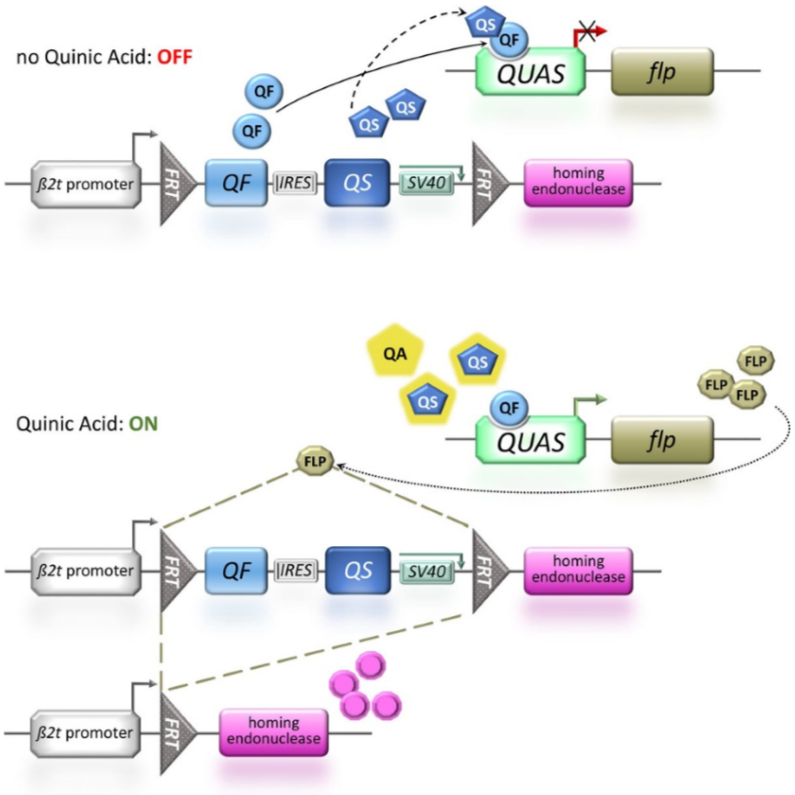

Figure 2 Reproductive sterility using a homing endonuclease controlled by the inducible Q-system in combination with site-specific recombination. The proposed reproductive sterility system is based on the inducible binary expression system Q [48], in which quinic acid (QA) acts as an inducer that hinders the repressor QS from complexing the transcriptional activator QF, which can activate its target genes by binding to a Q upstream activation sequence (*QUAS*). To generate male reproductive sterility systems the spermatogenesis-specific promoter of the *β2 tubulin (β2t)* gene can be suitably used to affect either the sperm itself or the progeny sired by the sperm. The Q system can be combined with a recombinase mediated transcription regulation system to render the induction of an effector gene expression permanent and independent of the presence of the inducer QA. In this dual system, QF drives the expression of a site-specific recombinase (FLP) that can in turn remove a *flp*-out cassette [57], which contains a transcriptional terminator (*SV40*) and is flanked by *flp* recombinant target sites (*FRTs*) in direct orientation. After the removal of the transcriptional terminator, the directed expression of an effector gene is mediated by the tissue-specific promoter 5' to the FRT. Since the Q system components are superfluous after the activation of the effector gene, they can also be placed into the *flp*-out cassette. To make sure that both components of the Q system are translated in a bi-cistronic messenger RNA, they will be separated by an internal ribosome entry site (IRES). A homing endonuclease targeting the progeny genome can be employed as an effector that would kill the progeny but not the sperm itself [34]. During regular rearing this male reproductive sterility would be kept in an OFF state, as at the absence of QA the repressor QS will mask QF and block its activation potential. Only after the addition of QA to the food in the release generation, QS will be inactivated and QF thereby allowed to activate the expression of the *flp* recombinase (FLP), which in turn would remove the Q system regulators and at the same time mediate the expression of the homing endonuclease that could block development of the next generation and thus cause male reproductive sterility.

placed accordingly to make sure that repressor QS will be in surplus to the activator QF.

In *D. melanogaster* it has been shown that FLP expression driven by the *β2 tubulin (β2 tub)* promoter is highly efficient to cause cassette flip-out during spermatogenesis leading to the transmission of the activated effector construct into the next generation [56,57]. Since the *β2 tub* promoter would also enable the generation of reproductive sterility systems [7], this promoter would be very suitable for such a complex system. Respective promoters have already been cloned from a number of different tephritid and mosquito species and functionally used for sperm marking purposes [59-61].

To cause reproductive sterility, finally an effector needs to be activated that either causes male sterility by sperm depletion, e.g. by expression of a cell death gene or a cell-specific toxin that is active in the cytoplasm only and has no trans-membrane movement abilities to protect adjacent tissue or predatory organisms [7,61]. However, as such sterile males would not transfer sperm

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to females, such females would continue to search further for sperm-providing wild type males. Therefore an effector that would kill the progeny but not the sperm would thus be much more suitable. This will allow for sperm development and transfer and therefore renders the females at least temporarily refractory to subsequent matings with wild type males. Such an effector could be a homing endonuclease (Figure 2) that does not affect spermatogenesis - thus producing functional sperm - but attacks the genome of the zygote or prevents the fusion of the male and female pro-nuclei [34]. This would serve as the best reproductive sterility mechanism as it would cause a dominant early embryonic lethality without affecting the sperm itself by stopping the development of the progeny at the very beginning. Moreover, a homing endonuclease would also be independent in its function from the proposed hyperactive pro-apoptotic gene suggested for the sexing system (Figure 1). However, it should be noted that for an applicable transgenic reproductive sterility system, 100% male sterility needs to be reached, which requires efficient *flp* recombinase repression in the absence of quinic acid and its effective induction in the presence of quinic acid as well as strong expression of a highly active homing endonuclease.

Partial redundancy of the female lethality and reproductive sterility systems

The described female lethality and reproductive sterility systems will in fact not be fully redundant, as only the female progeny of the released males will indeed have both lethality systems working. In the male progeny only the reproductive sterility providing the homing endonuclease will be active. Thus, rare strong resistance-mediating alleles might be selected in such male progeny and potentially lead to the accumulation of both the resistance allele and the transgenic lethality allele [22]. However, in case of direct linkage between the two lethality systems, which can be achieved by transgene modification based on site-specific recombination [62], the female lethality in the following generation would severely reduce the chance of accumulation of the lethality allele and thus reduce also the selection of the resistance allele. Since only resistant males would survive, they would be outcompeted by released susceptible SIT males [22].

Multifactorial reproductive sterility by an endonuclease causing chromosome shredding

Ideally the reproductive sterility system itself should be highly redundant to cause many different lethal mutations similar to the built-in redundancy of radiation-induced sterility [21]. To achieve this, it would be great to have a number of diverse endonucleases or endonuclease target sites causing chromosome shredding [63]. For this, we propose the employment of an endonuclease from the adaptive bacterial immune system using as essential component clustered regularly interspaced short palindromic repeats (CRISPR) [64,65], which allows bacteria to defend themselves against viruses they encountered before by recognizing and cutting the viral DNA sequences. For the human pathogen *Streptococcus pyogenes*, it could be shown that a single endonuclease, CRISPR-associated nuclease 9 (Cas9), is sufficient to cleave the target DNA [66]. Since it was shown that Cas9 can be directed to any 'protospacer' sequence followed by a protospacer-adjacent motif (PAM) that has only two required bases (NGG) [67] by using short guide RNAs (gRNAs) [68], this CRIPSR/Cas9 system has been successfully employed in many model and non-model organisms to generate gene knockouts and genome editing [69]. Recently a feature article on this emerging technology has discussed possible uses of the CRIPSR/Cas9 system in gene drives to alter wild populations [70].

By transgenic expression of several gRNAs using RNA polymerase III-dependent promoters, such as the *U6* snRNA promoter, it has been shown that the Cas9 endonuclease can actually be targeted to several diverse targets, which can lead to a mutagenesis rate of up to 100% [71,72]. By our proposed use of the *β2 tub* promoter, Cas9 will be highly expressed during spermatogenesis and the mRNA still be highly translated during spermiogenesis [73] to expose the sperm chromosomes to high amounts of the endonuclease (Figure 3). To cause chromosome shredding, several guide RNAs can be employed to direct the CRISPR/Cas9 endonuclaese to para-centromeric, sub-telomeric, and microsatellite sequences. The induced double strand breaks will lead to large chromosomal aberrations causing aneuploidies that will mediate multifactorial reproductive sterility.

In fact, one of the caveats of the Cas9 technology - the potential lack of specificity leading to off-target effects [74] - can serve as an additional advantage in the proposed use here, since it might lead to pleiotropic effects harming further genomic loci. Targeting many chromosomal locations will thus provide the intended redundancy bringing the transgene-induced reproductive sterility a step closer to the built-in redundancy of radiation-induced sterility [21].

Conclusions

The combination of a transgenic sexing system to meet the SIT requirement of male-only releases based on the repressible tTA directed expression system to create female-specific embryonic lethality using a sex-specifically spliced intron and a hyperactive pro-apoptotic gene (Figure 1) together with a reproductive sterility system based on a sperm-specifically expressed endonuclease

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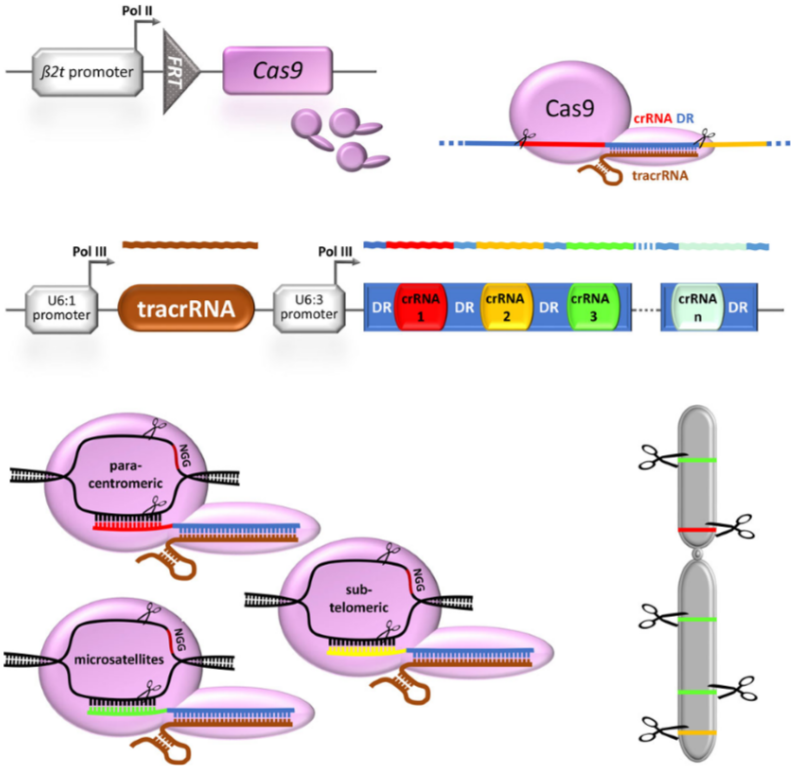

Figure 3 Multifactorial reproductive sterility based on the CRISPR/Cas9 system causing chromosome shredding. The bacterial derived Cas9 endonuclease will be expressed under the control of the *β2 tubulin (β2t)* promoter. Cas9 will be targeted to para-centromeric, sub-telomeric, and diverse macrosatellite sequences by guide RNAs, which are encoded by a CRISPR RNA (crRNA) array. This crRNA array as well as the *trans*-acting crRNA (tracrRNA) will be expressed under diverse RNA polymerase III promoters such as from the snRNA *U6* (U6:1, U6:3). In the crRNA array, the diverse crRNAs are separated by direct repeat sequences (DR) derived from the *Streptococcus pyogenes* CRISPR. The expressed Cas9 is loaded with tracrRNA and subsequently binds the crRNA array based on complementarity between tracrRNA and the DR sequences, thereby randomly selecting one of the crRNAs as a guide to produce a functional CRISPR/Cas9 endonuclease targeting the respective genomic loci [75], which will lead to double strand breaks causing chromosome shredding.

controlled by the inducible Q-system in combination with site-specific recombination (Figure 2) seems a promising approach. These two systems would not share any active components and the lethality would be mediated by completely independent processes. Therefore, cross-resistance to both lethality-mediating processes is extremely unlikely and resistance development would require at least two independent gene loci with the likelihood of co-existence and selection being significantly reduced [25]. It should be noted, however, that this redundancy is only partial as only the female progeny of respective released males will have both lethality systems at work. While this will still reduce the likelihood of accumulating transgenic lethal alleles and resistance alleles, we propose an additional level of redundancy for the reproductive sterility system using the CRISPR/Cas9 endonuclease system targeting several chromosomal locations to induce chromosome shredding in the sperm (Figure 3).

The insect strains carrying the combined transgenic female lethality and multifactorial reproductive male sterility systems would be reared on tetracycline containing food to suppress the female-specific lethality. The male reproductive sterility would not be activated yet, since the repressor QS would keep the system in an OFF state (Figure 4A). The adult flies of the pre-release generation would then be aged on tetracycline-free food (Figure 4B) in order to stop the suppression of the embryonic female-specific lethality in the next generation [29,41,42]. The release generation should then be grown also on tetracycline-free larval food in order to keep the embryonic sexing system on to produce males only: in the absence of tetracycline, the synthetic trans-activator tTA would activate a hyper-active pro-apoptotic gene that would lead to programmed cell death in the female embryos, as only the female-specific splicing of the *transformer* intron in this transcript results in the

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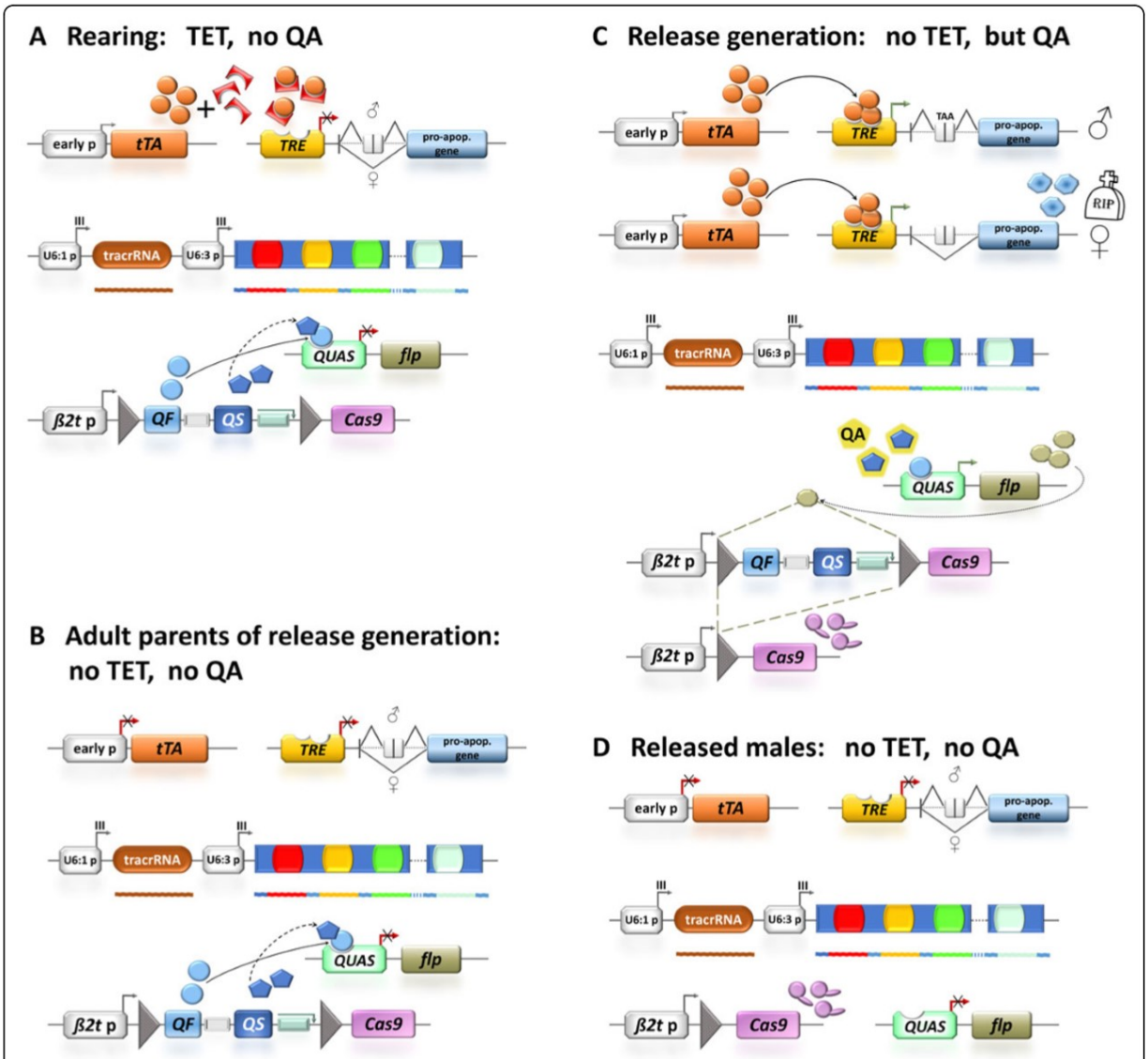

Figure 4 Rearing scheme for combined female lethality and multifactorial reproductive sterility systems. **A** Under regular rearing conditions, tetracycline (TET) is added to the food to repress the female lethality, quinic acid (QA) is not required for rearing. **B** The adult parents of the release generation will be changed to food without TET, still also without QA. This is necessary to avoid suppression of the early embryonic lethality in the next generation by maternally transferred TET to the oocyte. The female lethality system is still off, since the early embryonic promoter is not driving tTA at adult stages. **C** The release generation is then reared on food without TET but with added QA. Due to the lack of TET the female lethality system is switched on and the females die during early development. The QA leads to the activation of the Q system that leads to the expression of a site specific recombinase, which in turn mediates the spermatogenesis-specific expression of the Cas9 endonuclease by removing a recombination site-flanked spacer cassette. **D** The released males (no TET, no further QA) express high levels of the endonuclease Cas9 and multiple guide RNAs during spermatogenesis causing shredded chromosomes that will lead to lethal aneuploidy in the next generation.

production of an mRNA capable of translating the functional hyper-active pro-apoptotic protein (Figure 4C). The larval food for the release generation would, however, need to contain quinic acid to inactivate the repressor QS, which would then allow the activator QF to induce the expression of the *flp* recombinase gene, which then in turn would remove the Q system regulators and mediate the expression of the heterologous endonuclease Cas9 during spermatogenesis (Figure 4C). Released males (Figure 4D) would produce sperm with shredded chromosomes leading to lethal aneuploidy in the next generation similar to radiation-induced

reproductive sterility without suffering of somatic damages that cause reduced fitness.

A transgenic SIT approach using independent lethality systems would meet the 'redundant killing' criteria for suppression of resistance development and could therefore be employed in large scale long-term suppression programs.

Competing interests
EAW holds a patent on 'Universal Markers of Transgenesis'(United States Patent No. 6,518,481 B1)

Authors' contributions
EAW designed the project and wrote the first draft of the manuscript. KNE created the figures. All authors contributed to the conception of the project as well as critically revised and approved of the manuscript.

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3.2.3 Genetic engineering of a Killer-Sperm System to improve the Sterile Insect Technique

Current SIT male reproductive sterility approaches are collectively designed to generate males whose sperm transmits a dominant lethal transgene to their progeny that leads to embryonic lethality. However, female re-mating is a common behavior among insects that frequently leads to the dilution of transgenic with wild-type sperm, which can significantly reduce SIT effectiveness. To overcome this limitation, we here report on our progress towards the bioengineering of a novel and innovative killer-sperm based male reproductive sterility system as a proof-of-concept in *D. melanogaster*, in which males transfer a lethal factor along with their sperm that kills receiving females. Such a system will greatly improve SIT effectivity, as it not only guarantees male sterility but also restrains females from polyandrous mating and oviposition or blood sucking activities. While this project is still under development, we here present our advances made on the generation of functional tissue-specific driver lines, the evaluation of lethality factor candidates, and cloning of killer-sperm system effector constructs.

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STATUS:

Work in progress

AUTHOR CONTRIBUTIONS:

- Designing research and experiments [1]
- Cloning of driver and effector construct plasmids
- Germline transformation of *D. melanogaster,* including microinjections, screening, and individual backcrossing of G_0 flies
- Evaluation of driver strains
- Protein production and toxicity assays
- Preparing the figures
- Writing the manuscript

[1] together with I.M.C. and E.A.W.

Results

General concept of the killer-sperm system

The rationale of our killer-sperm system as a proof-of-concept in *D. melanogaster* is based on the expression of an inactive lethal factor in the reproductive tissue of bioengineered males, that is transferred into the female during copulation, where it will be activated to exert its cytotoxic effect shortly thereafter. For the feasibility of such a system, several indispensable prerequisites must be met, including the consecutive secretion of the effector protein by the expressing cells into the seminal fluid and its transportation into the female fly. With this in mind, we aim to hijack a small male-specific protein termed Sex-Peptide (SP; also known as ACP70A: accessory gland protein 70A) (Aigaki et al., 1991) by fusing the effector transgene to its C-terminus. SP is expressed in and secreted by the accessory gland main cells into the ejaculate, where it efficiently binds to the tail of sperm, ensuring its reliable transfer into the female (Kubli, 1992; Peng et al., 2005; Tsuda et al., 2015). Another requirement for our system, and an additional commonality to SP, is that our candidate toxin must remain inactive in the male and only become activated when entering the female reproductive tract. Interestingly, SP, as well as many known pre-toxins, require proteolytic processing by proteases for their activation (Peng et al., 2005). Hence, finding or bioengineering a pre-toxin that can be activated by the same protease that processes SP would allow us to meet this essential precondition. Lastly, the expression of such a SP-pro-toxin fusion gene must be conditional and specific to the tissue of the male reproductive tract. To this end, we contemplate to employ the TET-off binary expression system (Gossen and Bujard, 1992), and drive expression of the the *tetracycline-repressible transactivator* (*tTA*) by use of the 5' regulatory region of a gene specific to the accessory glands or ejaculatory duct, which will, under restrictive conditions, subsequently lead to the expression of the killer-sperm effector fusion transgene (Fig. 3.2.3-1).

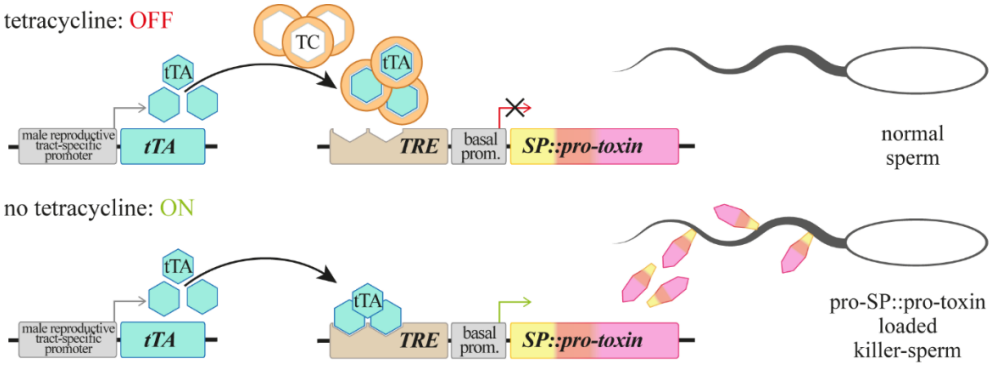

***Figure 3.2.3-1*: Schematic representation of the killed-sperm system.** The driver construct (left) comprises a male reproductive tract-specific promoter that initiates the expression of the tetracycline-repressible transactivator (tTA) e.g. in the accessory glands or ejaculatory duct. The effector construct (right) encompasses the *tTA response element* (TRE) combined with the *p-element* basal promoter that together control the expression of the *Sex-Peptide* (*SP*) – *pro-toxin* fusion effector transgene. Under permissive rearing conditions (top), i.e. in the presence of tetracycline (TET), the killer-sperm system is OFF, as TET prevents tTA from binding to the TRE-element, leading to the generation of normal, non-toxic sperm. Under restrictive release conditions (bottom), i.e. in the absence of TET, the killer-sperm system is ON, as tTA can now bind the TRE-element resulting in the expression of the *SP::pro-toxin* effector transgene, whose encoded protein will then bind to the sperm, ensuring its effective transport into the female during mating.

Generation of killer-sperm driver constructs and lines

For the tissue-specific expression of *tTA,* we generated two different killer-sperm driver constructs utilizing the 5' regulatory region of the *ACP70A* gene and the *ductus ejaculatorius peptide 99B* (*DUP99B*) gene (Saudan et al., 2002), which are highly and exclusively expressed genes in the main cells of the male accessory glands and the ejaculatory duct, respectively (Figure 3.2.3-3 B-C). Both driver constructs were cloned into a *piggyBac* donor vector that additionally contains a *PUb-DsRed* transformation/body marker (Handler and Harrell, 2001b), creating plasmids *KNE017 pBac{ACP70A-tTA_PUb-DsRed}* (Eckermann et al., 2018) and *KNE018 pBac{DUP99B-tTA_PUb-DsRed}*. These plasmids were subsequently used for *piggyBac*-mediated germline transformation, from which we obtained independent driver lines for each of both constructs *KNE017* and *KNE018*, of which six were homozygous viable and examined for their functionality.

Evaluation of tissue-specific tTA-expression in the killer-sperm driver lines

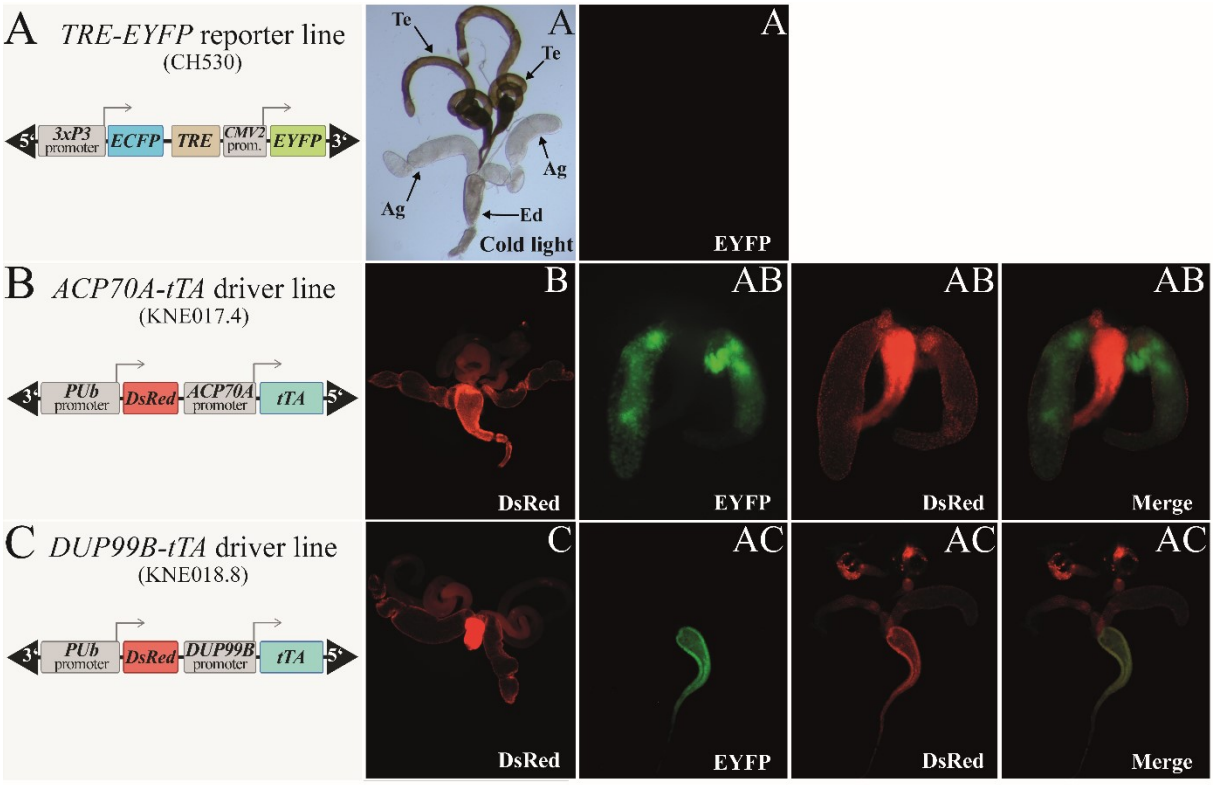

***Figure 3.2.3-2*: Evaluation of the killer-sperm driver lines by examining tTA-expression in the male reproductive tract.** Tissue-specificity of tTA expression in killer-sperm driver lines was assessed by crossing homozygous driver males **(B, C)** to female virgins of the **(A)** *TRE-EYFP* reporter line CH530 (Horn et al., 2003) under restrictive conditions. Double-heterozygous male offspring carrying the *ACP70A-tTA* **(AB)** or *DUP99B-tTA* **(AC)** driver construct showed the expected tTA-induced tissue-specific expression of the EFYP reporter protein in the accessory glands (AB) or ejaculatory duct (AC), respectively. | **Te:** testis, **Ag:** accessory gland; **Ed:** ejaculatory duct.

To test whether the driver lines express *tTA* in a tissue-specific manner, we individually crossed homozygous males from the six *ACP70A-tTA* (KNE017) and the six *DUP99B-tTA* (KNE018) driver lines to homozygous virgins of a *TRE-EYFP* reporter line, CH530, (Fig. 3.2.3-2 A; Horn et al., 2003) on a TET-free diet (Fig. 3.2.3-2). Dissected male reproductive tracts of double-heterozygous sons from several independent crosses were examined by epifluorescence microscopy. All displayed the expected tissue specific expression of the *EYFP* reporter gene. However, driver KNE017.4 (Fig. 3.2.3-2 B) and KNE018.8 (Fig. 3.2.3-2 C) gave rise to the strongest EYFP signal in the accessory glands or ejaculatory duct, respectively. These results demonstrate the functionality of the two *tTA* driver lines to initiate expression of a downstream *TRE*-based responder gene in a tissue-specific manner.

Finding the right pro-toxin for the killer-sperm system

As mentioned above, the toxin for our killer-sperm system is required to be a pre-toxin, which is inoperative in the male but will take effect after activation through proteolysis in the female, analogous to the processing of SP. *SP* mRNA encodes for a 55 aa precursor (pre-pro-Sex-Peptide), which contains a signal peptide that is removed during its co-translational translocation into the ER, resulting in the secretion of the 36 aa pro-Sex-Peptide (Fig. 3.2.3-3 A) (Chen et al., 1988; Kubli, 1996). With the aid of other proteins, pro- SP then binds N-terminally to the tail of the sperm, which facilitates its transfer into the female sperm storage organs after mating (LaFlamme et al., 2012; Peng et al., 2005; Ravi Ram and Wolfner, 2007; Tsuda et al., 2015). In this environment a, yet unknown, trypsin-like serine protease cleaves within the 'PWNR$^{\downarrow}$KP' SP protease recognition site (SP-PRS), and thereby releases the 29 aa C-terminal portion, which will then, as mature SP, bind to its cognate Sex-Peptide Receptor, triggering long-term post-mating responses (PMR) (Chapman et al., 2003; LaFlamme and Wolfner, 2013; Liu and Kubli, 2003; Peng et al., 2005; Yapici et al., 2008). Importantly, it has been shown that SP continues to be efficiently secreted when fused to another protein by its C-terminus (Eckermann, 2013; Minami et al., 2012; Villella et al., 2006), and is still capable of binding to the tail of sperm (Minami et al., 2012; Peng et al., 2005), which sets central preconditions for our approach.

Our first pro-toxin candidate is the pore-forming δ endotoxin Crystal 4A (Cry4A), which is a derivate of the large group of Crystal (Cry) insecticidal toxins, and is produced and aggregated by *Bacillus thuringiensis* (Bt) subsp. *israelensis* during sporulation (Yamagiwa et al., 2001). Depending on their individual target specificity, Bt-toxins are frequently applied in transgenic plants as a measure against feeding pests or in waterbodies as mosquitocides for vector control (Bravo et al., 2007; MacIntosh, 2010; Roh et al., 2007). Notably, Cry4A has been reported to be dipteran-specific (Yamagiwa et al., 1999), as well as being processed, similarly to SP, by a trypsin-like serine protease (Fig. 3.2.3-2 B)

(Angsuthanasombat et al., 2004), which may facilitate their simultaneous activation. The toxicity-activating step and mode of action of the 1180 aa pro-Cry4A occurs upon ingestion via three defined cleavages that take place within the insect midgut (Fig. 3.2.3-3 B), giving rise to an 178 aa (~20 kDa) N- and 460 aa (~45 kDa) C-terminal moiety that bind to and oligomerizes at the microvillus membrane of epithelial cells, generating a pore that leads cytolysis-mediated death of the insect larvae (Yamagiwa et al., 2001, 1999). However, while it is known that Cry4A effectively kills larvae of *Aedes*, *Anopheles* and *Culex* (Ben-Dov, 2014; De Barros Moreira Beltrão and Silva-Filha, 2007; Otieno-Ayayo et al., 2008), its insecticidal activity against drosophilids and tephritid fruit flies has not been investigated as yet.

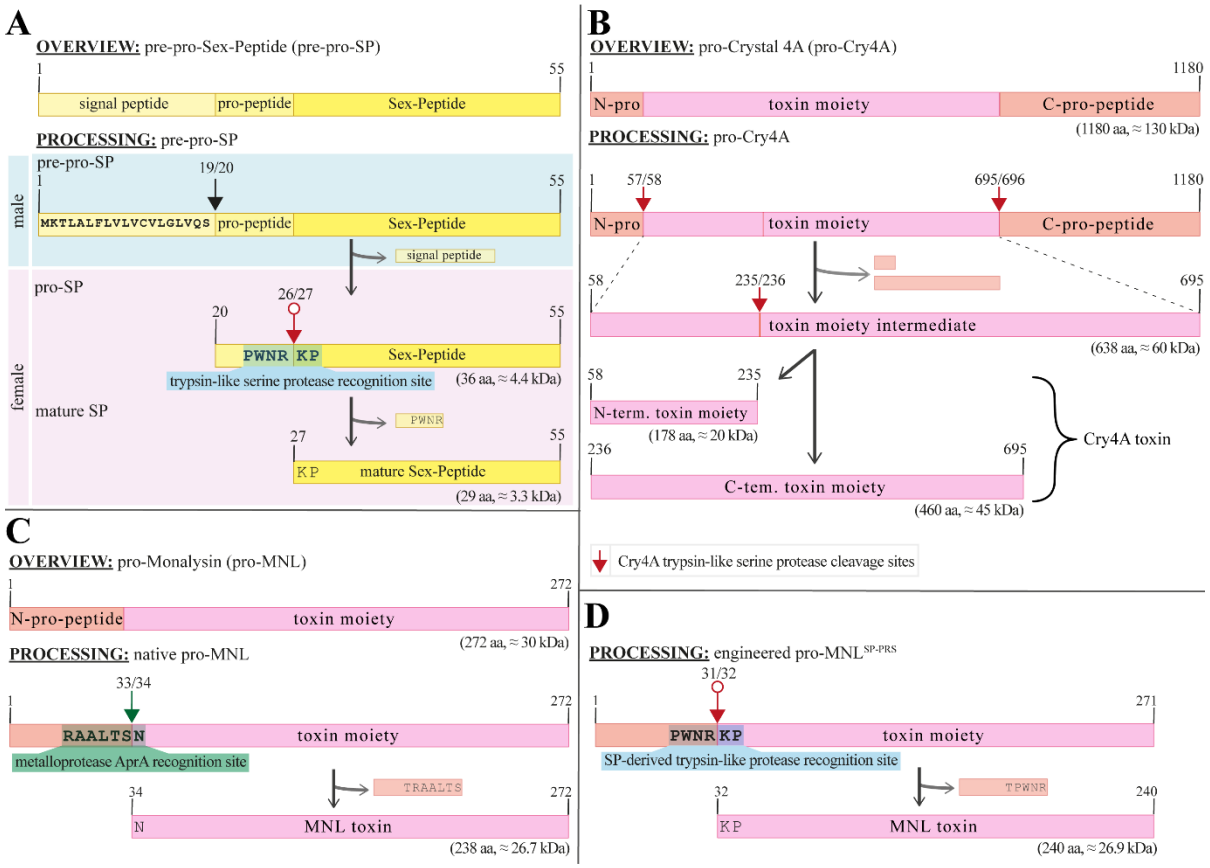

Figure 3.2.3-3: **Illustration of protein composition and specific proteolytic processing of the pre-pro-Sex-Peptide and pro-toxin candidates. (A)** *SP* encodes for a 55 aa pre-pro-peptide, comprising a hydrophobic N-terminal signal peptide, a pro-peptide, and C-terminal peptide hormone moiety. The signal peptide is clipped off by a signal peptidase during co-translational translocation at aa residues 19/20 (black arrow). After secretion and sperm-mediated transfer into the female the 36 aa pro-SP is cleaved by a presumably female reproductive tract-specific trypsin-like serine protease with the 'PWNRKP' recognition site (blue-shaded font) at residues 26/27 (red arrow with circle), releasing the 29 aa long mature SP hormone from the sperm. **(B)** The 1180 aa pro-Cry4A comprises an N- and C-terminal pro-peptide, which are both removed through proteolysis by a trypsin-like protease at the corresponding cleavage sites at residues 57/58 and 695/696 (red arrows), respectively. This gives rise to a 638 aa intermediate that consists of a smaller N- and larger C-terminal toxin moiety, which are subsequently detached via an additional cleavage of similar type between residues 235/236 (red arrow) and together form the activated Cry4A toxin. **(C)** In contrast, pro-MNL, with a total length of 272 aa, is significantly smaller compared to pro-Cry4A and contains a single metalloprotease recognition site instead (green-shaded font), which conjoins the short N-terminal pro-peptide with the toxin moiety. Proteolytic removal of the activity-inhibiting pro-peptide via cleavage by the metalloprotease AprA, within its 'RAALTSN' recognition site at residues 33/34 (green arrow), leads to the release of the operative MNL toxin. **(D)** Our engineered version of the *pro-MNL* gene encodes for altered variant of the pro-toxin, in which we replaced the sequence that encodes for the original metalloprotease recognition site ('RAALTSN') for the sequence that translates to the trypsin-like serine protease recognition site of SP (SP-PRS: 'PWNRKP'; blue-shaded font). This variant, which we termed pro-MNL$^{SP\text{-}SPR}$, should now ideally be exclusively processable by the same trypsin-like serine protease that cleaves pro-SP, and therefore facilitate an analogous female reproductive tract-specific activation of the MNL toxin.

In order to examine the insecticidal activity of Cry4A towards these genera we purified recombinant pro-Cry4A protein and performed a feeding-based toxicity assay on *D. melanogaster* and *C. capitata* larvae by adding different pro-toxin concentrations to their respective larval diet, increasing from 25 to 500 µg/ml (Fig. 3.2.3-4 B, C; Material & Methods 3.2.4.2.4). We applied the same pro-Cry4A ratios also to the rearing water of *Ae. aegypti* larvae, and additionally performed a "buffer only assay" to exclude that observed effects could be due to the co-increasing salt concentrations from the pro-toxin storage buffer. The results from the toxicity assay showed the expected toxicity of Cry4A towards *Ae. aegypti* larvae at the three highest pro-toxin concentrations (100 µg/ml, 250 µg/ml, 500 µg/ml), with no survivors at the end the observation period, and slightly milder effects at lower toxin levels (Fig. 3.2.3-4 A). Although, we could also notice some lethality at higher sodium chloride (NaCl) concentrations in the buffer-only assay, this cannot refute the monitored toxicity of Cry4A against *Ae. aegypti*. However, feeding the pro-toxin to larvae of *D. melanogaster* and *C. capitata* applying the same concentrations did not result in any detectable lethality (Fig. 3.2.3-3 B-C). Consequently, pro-Cry4A was dismissed as a potential pro-toxin for the development of the proof-of-principle killer-sperm system in *D. melanogaster* and future applications in *C. capitata.* For this reason, we were required to find a new pro-toxin candidate, which is already known to be toxic to *D. melanogaster* and still meets the requirements for our system.

With this end in mind, our attention was drawn to the more recently identified novel β-pore-forming exotoxin termed Monalysin (MNL), which is secreted as a 272 long pro-toxin (pro-MNL, ~30 kDa) by the *D. melanogaster* entomopathogen *Pseudomonas entomophila* (Opota et al., 2011). Post secretion, the toxin-activating processing of pro-MNL to its 238 aa (~26.5 kDa) mature form (Fig. 3.2.3-3 C) and its following mode of action is reminiscent to that of Cry4A and other pore-forming pro-toxins, as it also requires proteolytic cleavage before causing necrotic cell lysis by means of multimerization-based membrane pore formation (Blemont et al., 2013; Leone et al., 2015). Intriguingly, it was shown that pre-activated mature MNL induces high levels of lethality after injection into the body cavity of *D. melanogaster* adult flies, causes strong cytotoxicity when applied to *D. melanogaster* Schneider 2 and lepidopteran derived SF9 cells, and is also capable to form pores in artificial planar lipid bilayers (Dieppois et al., 2015; Opota et al., 2011). However, in contrast to the serine protease-mediated processing of pro-SP and pro-Cry4a, efficient proteolytic activation of pro-MNL through removal of the N-terminal pro-peptide is usually executed by the metalloprotease AprA (Fig. 3.2.3-3 C), which is abundantly co-secreted by *P. entomophila* (Liehl et al., 2006; Opota et al., 2011). Nevertheless, trypsin-based activational cleavage of pro-MNL has also been shown to be possible, albeit at lower efficiency (Opota et al., 2011).

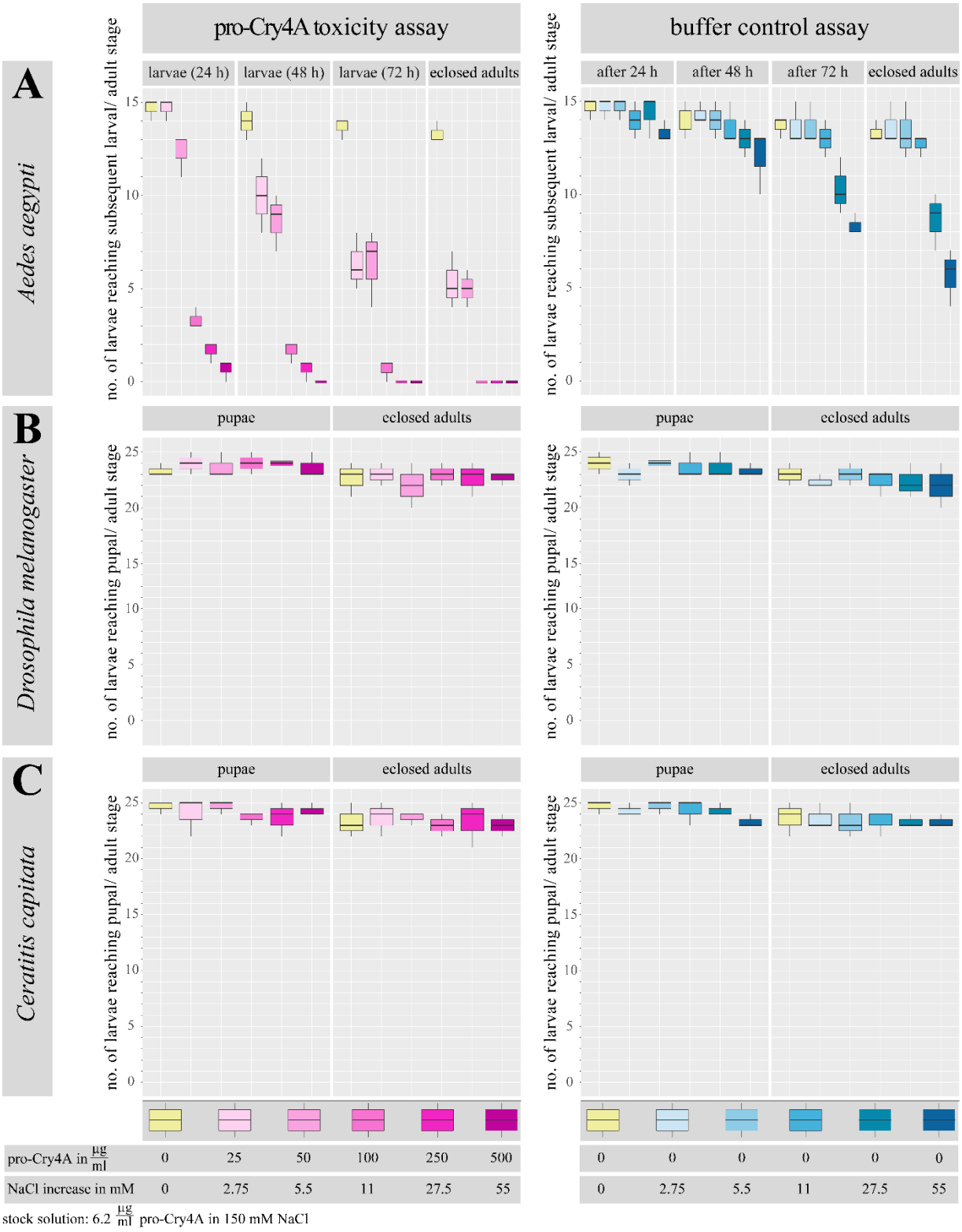

Figure 3.2.3-4: **Toxicity assay of pro-Cry4A against larvae of (A) *Ae. aegypti*, (B) *D. melanogaster*, and (C) *C. capitata*.** Shown is the effect of the Cry4A pro-toxin on the survival of larvae of different stages, pupae, and eclosed adults depending on pro-toxin concentration (left, magenta color gradation). Respective pro-toxin concentrations used in the experiments are indicated at the bottom (pro-Cry4A in µg/ml). In the buffer only control assay analogous salt concentrations without toxin were added (right, blue color gradation) and the relative NaCl increase is given at the bottom (NaCl in mM). **(A)** For *Ae. aegypti* 15 first or second instar larvae were exposed to varying concentrations of pro-Cry4A (25, 50, 100, 250, or 500 µg/ml) in rearing water. Toxicity of pro-Cry4A was assessed by counting alive larvae after 24, 48 and 72 h, and number of eclosed

adult mosquitos. Mean numbers from three replicates are shown as box plot. The survival rate decreased with increasing pro-toxin concentration, with no survivors and the three highest pro-toxin levels. Increase in salt concentration in buffer control also had effects on survival at the two highest NaCl levels. Although is effects was much less significant, it will have certainly contributed to the observed lethality. **(B-C)** In contrast, for *D. melanogaster* and *C. capitata* 25 first or second instar larvae (in three replicates) were placed on food containing the same pro-Cry4A concentrations as used in (A). Toxicity was assessed by number of larvae reaching pupal stage and number of eclosed adult flies. Mean numbers from three replicates are shown as box plot. No effect was found, at either of the pro-Cry4A or salt concentrations.

Generation of killer-sperm effector constructs and lines

For the generation of the killer-sperm effector constructs we created two different variants of the Sex-Peptide/ MNL effector fusion transgenes. The first variant is made up of the straightforward fusion of native *pro-MNL* to the C-terminus of *pre-pro-SP* from which the stop codon was removed (*pre-pro-SP::pro-MNL*, Fig. 3.2.3-5 A). Owing to the presence of the signal peptide and the sperm binding capabilities of the N-terminus of pro-SP, expression of this variant should lead to efficient secretion and sperm-mediated transfer of the pro-SP::pro-MNL effector fusion protein into the female (Fig. 3.2.3-1). However, since we cannot estimate whether the metalloprotease recognition site of pro-MNL would also be specifically and exclusively processed in the female reproductive tract equally to the SP-PRS of pro-SP – or might already be cleaved by other proteases earlier in the male, or potentially remains entirely unprocessed – we decided to engineer a version of *pro-MNL,* in which the sequence encoding for the original metalloprotease recognition site is replaced for the sequence that translates to the SP-PRS (*pro-MNL$^{SP\text{-}PRS}$,* Fig. 3.2.3-3 D, Fig. 3.2.3-5 B). Consequently, this engineered pro-MNL$^{SP\text{-}PRS}$ should now ideally be activated by the same mode of processing as pro-SP. Moreover, an additional SP-PRS was placed in between of the *pro-SP* and *pro-MNL$^{SP\text{-}PRS}$* coding sequences, which should facilitate the correct processing and release of mature SP (*pre-pro-SP:(SP-PRS):pro-MNL$^{SP\text{-}PRS}$*, Fig. 3.2.3-5 B,C). Subsequently, either of the two fusion transgene variants (*pre-pro-SP::pro-MNL* and *pre-pro-SP:(SP-PRS):pro-MNL$^{SP\text{-}PRS}$*) were independently cloned 3-prime of the *tetracycline-transactivator response element* (*TRE*) and *P-element* basal promoter into a *piggyBac* donor vector that also contains a *PUb-EGFP* germline transformation/ body marker (Fig. 3.2.3-5 A, B) and minimal *attachment P* (*attP*) ΦC31-Intergrase site, creating the killer-sperm effector plasmids *pBac{a_attP_TREp-SP::MNL_a_PUb-nls-EGFP}* and *pBac{a_attP_TREp-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP},* respectively (Material and Methods 3.2.4.2.3 C).

Following this, both effector plasmids were used for *piggyBac*-mediated germline transformation for the generation of *D. melanogaster* killer-sperm effector lines, which should afterwards be crossed to the positively evaluated *ACP70A-tTA* and *DUP99B-tTA* driver lines in order examine the overall functionality of our killer-sperm system. However, unfortunately, numerous rounds of micro-injections of several thousand embryos did not yield any transgenic lines for neither of the two effector constructs.

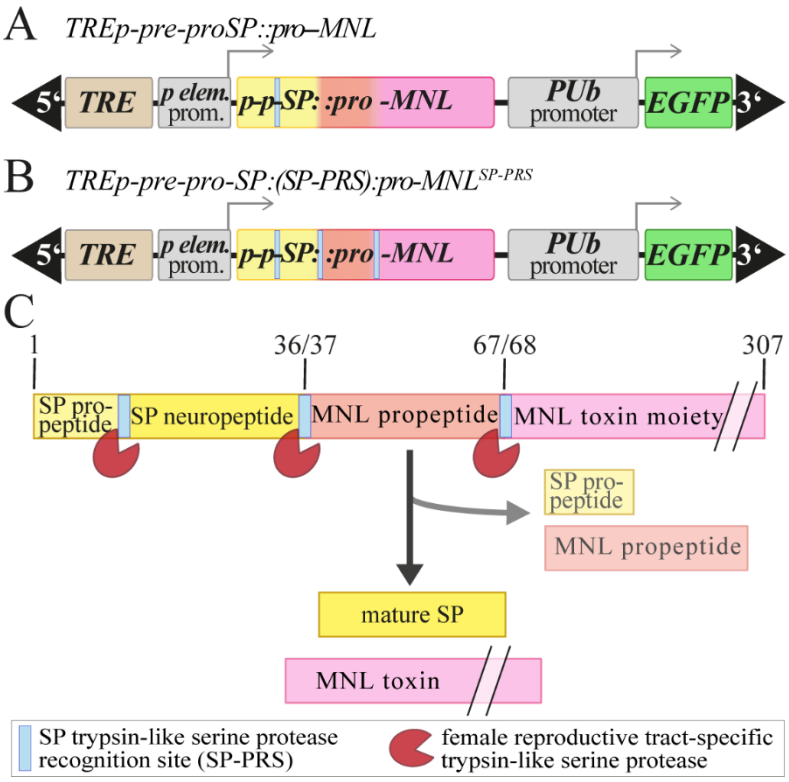

Figure 3.2.3-5: **Schematic representation of the two killer-sperm effector construct variants, containing (A) *pro-MNL*, or (B) the engineered version *pro-MNL*$^{SP\text{-}PRS}$, and (C) the predicted mode of processing of the engineered pre-pro-SP/pro-MNL$^{SP\text{-}PRS}$ fusion protein.** Shown are the two generated killer-sperm effector constructs (A-B) that both comprise 5- and 3-prime *piggyBac terminal inverted repeats* (5'and 3'), a *PUb-EGFP* transformation/ body marker, and the combination of the *tTA response element* (*TRE*) and basal *p- element* promoter that together control the expression of a effector *pre-pro-SP::pro-MNL* fusion transgene, in which (A) and (B) differ. **(A)** Effector construct containing the fusion transgene variant where the unaltered, *pro-MNL* CDS is fused to the stop-codon-less *pre-pro-SP* gene (*pre-pro-SP::pro-MNL*). The section that encodes for the pre-pro-SP-own trypsin-like serine protease recognition site (SP-PRS) is highlighted as a light blue bar. **(B)** Effector construct containing the second fusion transgene variant, where the engineered, modified *pro-MNL*$^{SP\text{-}PRS}$ version (described in Fig. 3.2.3-3 D) is employed and fused 3-prime to the stop-codon-less *pre-pro-SP* and the sequence that encodes for an additional SP-PRS, resulting in *pre-pro-SP:(SP-PRS):pro-MNL*$^{SP\text{-}PRS}$. Sections of the fusion gene that encodes for the SP-PRS are highlighted as a light blue bar. **(C)** Schematic depiction of the pro-SP:(SP-PRS):pro-MNL$^{SP\text{-}PRS}$ effector fusion protein post secretion (i.e. without signal peptide) as the gene product of the effector transgene of (B). Light blue bars indicate the three SP-PRSs - present in pro-SP, in pro-MNL$^{SP\text{-}SPR}$, and in between of the two fusion proteins -, which should be simultaneously and equally processed by a female productive tract-specific trypsin-like serine protease (dark red Pac-man), resulting in the removal of the N-terminal SP- and MNL pro-peptides and release of mature SP and the active MNL toxin.

Discussion

In this study, we delineated our progress on the development of a novel proof-of-concept killer-sperm system, as an advanced and more effective transgenic male reproductive sterility approach for the improvement of the Sterile Insect Technique. To create male flies that produce sperm that kills receiving females, we aimed to express a pro-SP::pro-toxin fusion protein via the conditional binary TET-off system in the male reproductive tract, which will be reliably transferred into the female during copulation based on the sperm-binding property of SP. To this end, we created an *ACP70A-tTA* and a *DUP99B-tTA* driver construct, which directs expression of the transactivator specifically to the cells of the accessory gland or ejaculatory duct, respectively (Aigaki et al., 1991; Saudan et al., 2002). Crosses of the subsequently established six *ACP70A-tTA* and six *DUP99B-tTA* driver lines to the *TRE-EYFP* reporter line (CH530)

(Horn et al., 2003) confirmed the expected production of tTA in the respective tissue, verifying their applicability for the killer-sperm system. The first pro-toxin candidate for usage in our pro-SP::pro-toxin fusion effector construct was the BTI pro-toxin pro-Cry4A, which was selected for its characteristics to be dipteran-specific and being activated by a trypsin-like serine protease, analogous to SP. However, the toxicity assay, in which we applied purified recombinant pro-Cry4A to larvae of *D. melanogaster* and *C. capitata,* did not show any noticeable lethality. We, therefore, revised our molecular design and selected the recently identified pro-MNL from *P. entomophilia* for utilization as pre-toxin in our planned fusion effector construct, as it has already been demonstrated to be highly toxic to *D. melanogaster* (Opota et al., 2011). However, in contrast to pro-SP and pro-Cry4A, regular activation of native pro-MNL occurs via a *P. entomophila*-endogenous metalloprotease at a metalloprotease recognition site. Based on this, it remains unclear if pro-MNL will also be exclusively processed in the designated female reproductive tract post transfer. To be prepared to provide a suitable response to this potential problem, we decided to generate an engineered variant of pro-MNL (pro-MNL$^{SP\text{-}PRS}$), in which we exchanged the metalloprotease recognition site for the SP-derived serine protease recognition site (SP-PRS), which should ideally copy the female reproductive tract-specific proteolytic activation of pro-SP to pro-MNL. The presumed mechanism by which premature proteolytic cleavage of pro-SP in the seminal fluid is prevented, is based on the presence of evolutionary mutated, non-catalytic serine protease homologs that function as natural inhibitors by binding to the recognition sites and thereby shielding them from untimely activation through active proteases (Findlay et al., 2014; LaFlamme and Wolfner, 2013). Another potential advantage of fusing the engineered pro-MNL$^{SP\text{-}PRS}$ to pro-SP, is that the repetitive presence of the same recognition site might increase the likelihood of common processing by the same protease molecule in quick succession, rather than when dependent on different proteases.

However, considerable efforts to generate lines for either of our *TREp-pre-pro-SP::pro-MNL* and *TREp-pre-pro-SP:(SP-PRS):pro-MNL*$^{SP\text{-}PRS}$ effector constructs via micro-injection-based germline transformation have failed to produce even a single transgenic line. Based on the great number of injected embryos (> 900/ effector construct), back-crossed G_0 flies, screened F_1 individuals, and our usually attained transformation rates using the hyperactive *piggyBac* transposases (Eckermann et al., 2018; chapter 3.1), we effectively rule out that this result could be due to the sheer absence of transformation events. As the *TREp* element has already been employed for the conditional expression of lethal effector constructs in other systems, for which there was no indication of a basal promoter activity (Horn and Wimmer, 2003), we suspect that genomic position effects could be responsible for a leaky expression of the deadly pre-pro-SP::pro-toxin effector protein in tissues other than those intended, in which other potent proteases maybe active or less tightly regulated compared to the female reproductive tract. Consequently, this could have impeded the development of successfully transformed individuals. In order to shield the

inserted effector construct from *cis*-regulatory influences of surrounding euchromatin, or the *trans*-regulatory action of distant enhancer elements at its site of integration, the utilization of insulator sequences that flank the effector construct, could be a solution to remedy this problem (Kaundal et al., 2014; Wallrath and Elgin, 1995; Wilson et al., 1990). The *gypsy* transposon or chicken *β-globulin* 5'HS4 insulator element belong to the best studied (Chung et al., 1993; Markstein et al., 2008; Sarkar et al., 2006; Scott et al., 1999) and most frequently used insulators within the community of molecular entomologists (Horn and Wimmer, 2003; Schetelig et al., 2009a; Yan and Scott, 2015; Zhao et al., 2020). For that purpose, we have already proceeded to re-clone both effector constructs into *piggyBac* transformation vectors containing either *gypsy* or 5'HS4 insulator elements, which will subsequently be used for micro-injections in near future.

However, more recent findings in the complex activation and regulation of seminal fluid proteins (SFPs) have also identified the presence of a variety of metalloproteases in the seminal fluid (Avila et al., 2011; LaFlamme et al., 2014; Sitnik et al., 2014). The best studied is the Seminal Metalloprotease-1 (Semp1), which itself is being activated by a two-step mechanism that requires both male and female components to be fully operational (LaFlamme et al., 2014). Following the complete activation of Semp-1 in the female, Semp-1 has been shown to proteolytically process the prominent SFPs ovulin and Acp36DE, which are required for the release of eggs and appropriate sperm storage, respectively (LaFlamme et al., 2014). These findings do not only increase the probability of native pro-MNL to be processed in this alien context, but also enlarges the repertoire of exploitable specific protease recognition sites that are known to be exclusively processed in the female reproductive tract. Furthermore, the proposed approach might achieve a broader applicability also in other, SP-lacking non-drosophilid species by creating an SP-independent, simplified killer-sperm system, in which the pro-toxin is merely equipped with an N-terminal signal peptide for orderly eukaryotic secretion and, if necessary, a suitable protease recognition site for appropriate activation. In this manner, the pre-toxin would simply be transferred into the female along with the other SFPs without being previously attached to the sperm. In preparation for this, we have already purified recombinant pro-MNL protein to test the susceptibility of actual pest species, such as *C. capitata* and *D. suzukii*, to the MNL toxin.

Material and Methods

For materials and methods please see section 3.2.4.

Acknowledgements

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Supplementarty Information

Sequence of the *pre-pro-SP:(SP-PRS):pro-MNL*$^{SP\text{-}PRS}$ fusion gene (GenBank format):

```
LOCUS       pre-pro-SP:(SP-PRS):pro-MNL^SP-PRS      996 bp    DNA     linear
DEFINITION  pre-pro-SP:(SP-PRS):pro-MNL^SP-PRS
ORGANISM    D. melanogaster/ P. entomophilia
FEATURES             Location/Qualifiers
  signal_peptide     1..57
                     /label="signal peptide"
     CDS             1..165
                     /label="pre-pro-Sex-Peptide (w/o stop codon)"
     Pro-peptide     58..78
                     /label="pro-peptide"
  Recognition_Site   67..84
                     /label="SP-PRS"
     Protein         79..165
                     /label="mature Sex-Peptide"
  Recognition_Site   166..183
                     /label="SP-PRS"
     CDS             184..996
                     /label="pro-MNL(SP-PRS)"
     Pro-peptide     184..273
                     /label="pro-peptide"
  Recognition_Site   262..279
                     /label="SP-PRS"
     Toxin           274..996
                     /label="MNL toxin"
ORIGIN
1   atgaaaactc tagcactatt cttggttctc gtttgcgtac tcggcttggt ccagtcctgg
61  gaatggccgt ggaataggaa gcctacaaag tttccaattc caagccccaa tcctcgtgat
121 aagtggtgcc gacttaattt ggggcccgcc tggggtggaa gatgtccgtg gaataggaag
181 cctacgatca aggaagagct gggccagcct caaagccatt cgatcgaact ggacgaggtg
241 agcaaggagg ccgcaagtac gccgtggaat aggaagccta acctgtctgg ccgcttcgac
301 cagtacccga ccaagaaggg cgactttgcg atcgatggtt atttgctgga ctacagctca
361 cccaagcaag gttgctgggt ggacggtatc actgtctatg gcgatatcta catcggcaag
421 cagaactggg gcacttatac ccgcccggtg tttgcctacc tacagtatgt ggaaaccatc
481 tccattccac agaatgtgac gaccaccctc agctatcagc tgaccaaggg gcatacccgt
541 tccttcgaga ccagtgtcaa cgccaagtac agcgttggcg ccaacataga tatcgtcaac
601 gtgggttcgg agatttccac cgggtttacc cgcagcgagt cctggtccac cacgcagtcg
661 ttcaccgata ccaccgagat gaaggggcca gggacgttcg tcatttacca ggtcgtgctg
721 gtgtatgcgc acaacgccac ctcggcaggg cggcagaatg ccaatgcctt cgcctacagc
781 aaaacccagg cagtgggctc gcgggtggac ttgtactact tgtcggccat tacccagcgc
841 aagcgggtca tcgttccgtc gagcaatgcc gtcacgccgc tggactggga tacggtgcaa
901 cgcaacgtgc tgatggaaaa ctacaaccca ggcagtaaca gcggacactt cagcttcgac
961 tggagtgcct acaacgatcc tcatcgccgt tattga
//
```

3.2.4 Material and Methods for the Killed- and Killer-Sperm System

3.2.4.1 Insect Strains & Cell Culture

3.2.4.1.1 *Ceratitis capitata*

Germline transformation experiments and toxicity feeding assays in *C. capitata* were performed with the *Egypt-II (EgII)* wild-type strain which was provided by the FAO/IAEA Agriculture and Biotechnology Laboratory (Entomology Unit, Seibersdorf, Austria). For the Killed-Sperm System generated #1413 *Ccβ2tub-tTA* driver strains (Eckermann et al., 2018; chapter 3.1) were crossed to the 1247_f1m2 *TREhs34-hid*^{Ala5} effector strain (Schetelig et al., 2009a). Strains were kept under standard relaxed artificial rearing conditions (Saul, 1982).

3.2.4.1.2 *Drosophila melanogaster*

Germline transformation experiments were performed with the *D. melanogaster Oregon-R* (*OreR*) wild-type strain (Lindsley and Grell, 1968). Double-balancer strain $w, \frac{wg^{Sp-1}}{CyO\ p\{Act-GFP\}}; \frac{Dr^1\ ry^{506}}{TM6B\ p\{Dfd-EYFP\}\ Tb^1\ Sb^1\ ca^1}$ (a gift from Marita Büscher, University of Göttingen) was used for crosses to identify on which chromosome an inserted transgene was located. Single-balancer stains $w^-; \frac{wg^{Gla-1}}{CyO}; \frac{+}{+}$ and $w^-; \frac{+}{+}; \frac{TM3\ Ser^1}{MKRS\ Sb^1}$ (gifts from Sigrid Hoyer-Fender, University of Göttingen) were used for crosses to balance or homozygous a transgene on chromosome 2 or 3, respectively. *TRE-EYFP*-Reporter strains CH530 $\left(w^-; \frac{p\{3xP3-CFP;TRE-EYFP\}}{p\{3xP3-CFP;TRE-EYFP\}}; \frac{MKRS}{TM2}\right)$ and CH509 $\left(w^-; \frac{wg^{Sp-1}}{CyO}; \frac{p\{3xP3-CFP;TRE-EYFP\}}{p\{3xP3-CFP;TRE-EYFP\}}\right)$ were used for crosses to various driver strains to test tissue-specific *tTA* expression (Horn et al., 2003). All strains were kept under standard relaxed artificial rearing conditions (Roberts, 1998).

3.2.4.1.3 *Aedes aegypti*

The *Ae. aegypti* Rockefeller ("ROCK") wild-type strain (Kuno, 2010) was a kind gift from Joachim Schachtner (Philipps-University Marburg) and was obtained as eggs. Hatched larvae were only used for the Cry4A toxicity assay. Eggs and larvae were treated as previously described with the modification of constant light instead of a 16/8 hours (light/dark) cycle and larvae were fed with a mixture of flake food (*Tetra Rubin, Tetra GmbH*, Melle, Germany) (Hays and Raikhel, 1990). Eclosed adult flies were euthanized after data collection.

3.2.4.1.4 *D. melanogaster* Schneider 2 Cell Culture

S2 cells were plated in 6 ml of S2 cell culture medium containing 90% *Drosophila* Schneider's S2 Medium, 10% heat-inactivated FBS and 0.005% PenStrep [10.000 units] (*Gibco/ Thermo Fisher Scientific*, Waltham U.S.A.) at 25 °C in a 25 cm^3 air-tight flask. Circa once per week when S2 cells reached 70-80% confluency S2 cells were split 1:10 with fresh S2 cell culture medium prior to transfection procedures (*Corning Incorporated*, New York, U.S.A.) (Buster et al., 2010; Johansen et al., 1989; Schneider, 1972). The S2 cells were a kind gift from Andreas Wodarz, University of Cologne.

3.2.4.2 Molecular Biology

Unless mentioned otherwise, standard protocols were followed (Sambrook and Russell, 2001). Likewise, standard buffers and solutions were prepared and used as described in Sambrook and Russell (2001) or *Lab FAQs 3rd edition* (*Roche Diagnostics*, Rotkreuz, Switzerland).

3.2.4.2.1 Cloning

Restriction endonucleases, T4 DNA Ligase and T7 DNA Ligase were, unless stated otherwise, obtained from *New England Biolabs* (Ipswich, U.S.A.) and used according to the manufacturer's protocol. Besides traditional restriction endonuclease-based cloning, assembly of several constructs was performed with the *In-Fusion® HD Cloning System* (*Takara Bio Europe/ Clontech*., Stain-Germain-en-Laye, France) following the manufacturer's instructions. For PCR-reactions, the Phusion® High-Fidelity DNA Polymerase system (*New England Biolabs*, Ipswich, U.S.A.) was used. Unless mentioned otherwise, annealing temperatures for oligonucleotides were calculated, as recommended, with *NEB Tm Calculator* (*New England Biolabs*, Ipswich, U.S.A.). Oligonucleotides were obtained from *Eurofins Scientific SE* (Luxembourg) and prior to order analyzed for hairpins and self-dimerization with *OligoAnalyzer 3.1* (*Integrated DNA Technologies*, Coralville, U.S.A.). Sub-cloning of DNA fragments and pre-assembly of constructs was done in pCR™II vector (*Invitrogen/ Thermo Fisher Scientific*, Waltham, U.S.A.). All cloned constructs were verified by sanger sequencing (*SEQLAB – Sequence Laboratories Göttingen GmbH*, Göttingen, Germany) prior to further use.

3.2.4.2.2 Cloning of plasmids for the Killed-Sperm System

***pBac{Ccß2tubulin-tTA_PUb-DsRed}* - (#1413)**

The plasmid #1413 was cloned as described in subchapter 3.1 (Eckermann et al., 2018) together with, and under supervision of Christian E. Ogaugwu (Eckermann, 2013).

***pXLII{Dmß2tubulin-tTA_PUb-DsRed_attP}* - (KNE007)**

The plasmid KNE007 was cloned as described in subchapter 3.1 (Eckermann et al., 2018) under my supervision together with Mohammad KaramiNejadRanjbar (KaramiNejadRanjbar, 2014).

3.2.4.2.3 Cloning of plasmids for the Killer-Sperm System

a) Male reproductive tract-specific tTA driver constructs

***pXLII{ACP70A-tTA_PUb-DsRed_attP}* - (KNE017)**

KNE017 was cloned as described in subchapter 3.1 (Eckermann et al., 2018).

***pXLII{DUP99B-tTA_PUb-DsRed_attP}* - (KNE018)**

For KNE018 the 1 kb *DUP99B* upstream region plus 5'UTR was amplified from genomic DNA with primers *DP99AgeIF/ DP99NheIR* (Table 3.2.4 A), which added an *Age*I and *Nhe*I cutsite to the 5' and 3' of the amplicon, respectively. Crude genomic DNA isolation was performed as described previously (Horn and Wimmer, 2003), with an increased volume of the squishing buffer to 100 µl since a male *D. melanogaster (Oregon-R)* head was used instead of embryos. The *AgeI_Dm-DUP99B_NheI* fragment and KNE007 plasmid were then restriction digested with *Age*I and *Nhe*I and subsequently ligated.

b) Sex-Peptide secretion test constructs for cell culture

***pJFRC7[20XUAS_Ac5c-mCherry]* - (KNE008)**

KNE008 was generated described in subchapter 3.1 (Eckermann et al., 2018).

***pJFRC7[20XUAS_Ac5c-SP::mCherry]* - (KNE009)**

KNE009 was generated assembling the following DNA fragments with the *In-Fusion® HD Cloning System* (Eckermann, 2013):

(i) backbone: The vector *pJFRC7-20XUAS-IVS-mCD8::GFP* (Pfeiffer et al., 2010) was digested with *Aat*II/*Bam*HI which removed the *IVS-mCD8::GFP* fragment. *pJFRC7-20XUAS-IVS-mCD8::GFP* was a gift from Gerald Rubin (Addgene plasmid # 26220).

(ii) insert: The ~0.3 kb *Actin5C* regulatory region was amplified from *p[Ac5C-Gal4]* with primers *IC102* and *IC30* (Table 3.2.4 A). *p[Ac5C-Gal4]* was a kind gift from Andreas Wodarz, University of Cologne.

(iii) insert: The *SP* CDS was amplified from *D. melanogaster* male cDNA with primers *IC10* and *IC93* (Table 3.2.4 A).
For the generation of the cDNA, at first, total RNA was isolated from six abdomens of *D. melanogaster* males using the Tissue & Insect RNA MicroPrep™ Kit (*Zymo Research Corporation*, Irvine U.S.A.). Prior to reverse transcription RNA was treated with TURBO™ DNase (*Ambion™/ Thermo Fisher Scientific*, Waltham, U.S.A.), before the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (*Thermo Fisher Scientific*, Waltham, U.S.A.) was used to perform reverse transcription. Template samples in which the reverse transcriptase was omitted from the single strand synthesis reaction were included as control RT-PCR.

(iv) insert: The *mCherry* CDS was amplified from *pcDNA3.1/hChR2(H134R)-mCherry* (Zhang et al., 2007) with primers *IC86* and *IC91* (Table 3.2.4 A). *pcDNA3.1/hChR2(H134R)-mCherry* was a gift from Karl Deisseroth (Addgene plasmid # 20938).

c) Killer-Sperm *SP::MNL*-fusion effector constructs

pBac{a_attP_TREp-SP::MNL_a_PUb-nls-EGFP} - (KNE033)

The plasmid KNE033 was generated by a series of sub-cloning steps. Intermediate constructs (i): KNE022 and (ii): KNE024 were created with the *In-Fusion® HD Cloning System.*

(i) ***pCRII[a_attP_TREp-SP::MNL] -* (KNE022):** The *a_attP_TREp* fragment was amplified with the primer-pair *IF_TRE_F/ IF_TRE_R* (Table 3.2.4 A) from plasmid #1262 *pBac{>fa_attP_f_TREp-hidAla5_a>_PUb-nls-EGFP}* (Schetelig et al., 2009a). The second insert comprised the *SP* CDS (w/o stop-codon) and was generated via PCR with primers *IF_SP::MNL_F* and *IF_SP::MNL_R* (Table 3.2.4 A) and the plasmid KNE009 *pJFRC7[20XUAS_Ac5c-SP::mCherry]* as template. The *MNL* CDS was amplified from pETG-20A-*Monalysin* (Opota et al., 2011) with primers *IF_MNL_C1_F/ IF_MNL_pCRII_R* (Table 3.2.4 A) pETG-20A-*Monalysin* was a kind gift from Bruno Lemaitre (Ecole Polytechnique Fédérale Lausanne) and Alain Roussel (Architecture et function des macromolecules biologiques, Marseille). The *pCRII* vector served as backbone and was digested with *Eco*RV.

(ii) ***pCRII[a_attP_TREp-SP::MNL_SV40_a] - (*KNE024):** The *a_attP_TREp-SP::MNL* fragment was PCR amplified from plasmid KNE022 *pCRII[a_attP_TREp-SP::MNL]* with primers *IF_TRE_F/ IF_MNL_R* (Table 3.2.4 A). The insert *SV40_a* #1247 was amplified from plasmid #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) with the primer pair *IF_SV40_F/ IF_SV40_AscI_R* (Table 3.2.4 A). The *pCRII* vector served as backbone and was digested with *Eco*RV.

(iii) ***pBac{a_attP_TREp-SP::MNL_a_PUb-nls-EGFP} - (KNE033):*** The fragment comprising *attP_TREp-SP::MNL_SV40* was generated from KNE024 via *Asc*I restriction. It was cloned in #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) via *Asc*I, creating *pBac{a_attP_TREp-SP::MNL_a_PUb-nls-EGFP}*.

pBac{a_attP_TREhs43-SP::MNL_a_PUb-nls-EGFP} - (KNE034)

The plasmid KNE034 was generated by a series of sub-cloning steps. Intermediate constructs (i): KNE023 and (ii): KNE025 were created with the *In-Fusion® HD Cloning System.*

(i) ***pCRII[a_attP_TREhs43-SP::MNL] -* (KNE023):** The *a_attP_TREhs43* fragment was amplified with the primer-pair *IF_TRE_F/ IF_TRE_R* (Table 3.2.4 A) from plasmid #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a). The second insert comprised the *SP* CDS (w/o stop-codon) and was generated via PCR with primers *IF_SP::MNL_F* and *IF_SP::MNL_R* (Table 3.2.4 A) and the plasmid KNE009 *pJFRC7[20XUAS_Ac5c-SP::mCherry]* as template. The *MNL* CDS was amplified from pETG-20A-*Monalysin* (Opota et al., 2011) with primers *IF_MNL_C1_F/ IF_MNL_pCRII_R* (Table 3.2.4 A). pETG-20A-*Monalysin* was a kind gift from Bruno Lemaitre (Ecole Polytechnique Fédérale Lausanne) and Alain Roussel (Architecture et function des macromolecules biologiques, Marseille). The *pCRII* vector served as backbone and was digested with *Eco*RV.

(ii) ***pCRII[a_attP_TREhs43-SP::MNL_SV40_a] - (*KNE025):** The *a_attP_TREhs43-SP::MNL* fragment was PCR amplified from plasmid KNE023 *pCRII[a_attP_TREp-SP::MNL]* with primers *IF_TRE_F/ IF_MNL_R* (Table 3.2.4 A). The insert *SV40_a* #1247 was amplified from plasmid #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) with the primer pair *IF_SV40_F/ IF_SV40_AscI_R* (Table 3.2.4 A). The *pCRII* vector served as backbone and was digested with *Eco*RV.

(iii) ***pBac{a_attP_TREhs43-SP::MNL_a_PUb-nls-EGFP} - (KNE034):*** The fragment comprising *attP_TREhs43-SP::MNL_SV40* was excised from KNE025 via *Asc*I restriction. It was cloned in #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) via *Asc*I, creating *pBac{a_attP_TREhs43-SP::MNL_a_PUb-nls-EGFP}*.

***pBac{a_attP_TREp-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}* - (KNE031)**

For the generation of plasmid KNE031 the precursor construct KNE027 had to be created first.

(i) ***pCRII[a_attP_TREp-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_SV40_a] - (KNE027):*** At first, KNE024 *pBac{a_attP_TREp-SP::MNL_a_PUb-nls-EGFP}* was sequentially digested with *Sma*I (at 25°C) and *EcoR*V (at 37°C), removing the fused *SP::MNL* CDS, which was subsequently replaced by the synthesized *SP:(SP-RS):MNL$^{SP\text{-}RS}$* gBlock (*Integrated DNA Technologies*, Coralville, U.S.A.) via *In-Fusion*® cloning. The gBlock comprised the for the *In-Fusion*® reaction required 5' and 3' overlapping sequences and contained the modified *SP::MNL*-fusion CDS with two additional *SP* protease recognition sites (*SP-PRS*).

(ii) ***pBac{a_attP_TREp-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}* - (KNE031):** The fragment comprising *attP_TREp-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_SV40* was excised from KNE027 via *Asc*I restriction. It was cloned in #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) via *Asc*I, creating *pBac{a_attP_TREp-SP:(SP-PRS): MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}*.

***pBac{a_attP_TREhs43-SP:(SP-PRS): MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}* - (KNE032)**

For the generation of plasmid KNE033 the precursor construct KNE028 had to be created first.

(i) ***pCRII[a_attP_TREhs43-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_SV40_a] - (KNE028):*** At first, KNE025 *pBac{a_attP_TREhs43-SP::MNL_a_PUb-nls-EGFP}* was sequentially digested with *Sma*I (at 25°C) and *EcoR*V (at 37°C), removing the fused *SP::MNL* CDS, which was subsequently replaced by the synthesized *SP:(SP-PRS):MNL$^{SP\text{-}PRS}$* gBlock (*Integrated DNA Technologies*, Coralville, U.S.A.) via *In-Fusion*® cloning. The gBlock comprised the for the *In-Fusion*® reaction required 3' and 5' overlapping sequences and contained the modified *SP::MNL*-fusion CDS with two additional *SP* cleavage sites (*SP-CS*).

(ii) ***pBac{a_attP_TREhs43-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}* - (KNE032):** The fragment comprising *attP_TREhs43-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_SV40* was excised from KNE028 via *Asc*I restriction. It was cloned in #1247 *pBac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-nls-EGFP}* (Schetelig et al., 2009a) via *Asc*I, creating *pBac{a_attP_TREhs43-SP:(SP-PRS):MNL$^{SP\text{-}PRS}$_a_PUb-nls-EGFP}*.

pBac{>fa_attP_TREp-SP:(SP-PRS):MNL^{SP-PRS}_a>>_PUb-nls-EGFP} **(KNE035)**

KNE035 was generated with the PCR-based and restriction-free mega-priming cloning strategy as previously described (Unger et al., 2010) with the below documented modifications.

(i) A PCR reaction with overhang-primers *Gib_SP:MNL_Ins_F*/ *Gib_SP:MNL_Ins_R* (Table 3.2.4 A) using KNE027 *pCRII[a_attP_TREp-SP:(SP-PRS):MNL^{SP-PRS}_SV40_a]* as template was performed which yielded the *attP_TREp-SP:(SP-PRS):MNL^{SP-PRS}* DNA fragment. The overhangs of the primers added an extra 30 bp to the 5' and 3' end of the amplicon which were overlapping with the sequences 5' and 3' of the *Asc*I restriction site in the destination vector *pBac{>**fa**>_PUb-nls-EGFP}*.

(ii) For the mega-priming PCR reaction the *KOD Xtreme^{TM} Hot Start DNA Polymerase* (*Merck Millipore*, Billerica, U.S.A.) was used, following the manufacturer's protocol for "Amplification of Long or Difficult DNA Targets" with the following modifications: instead of primers ~250 ng of the in (i) generated PCR fragment *attP_TREp-SP:(SP-PRS):MNL^{SP-PRS}* were used together with ~50 ng of the plasmid #1223 as template. Subsequently, *Dpn*I was supplemented to remove parental #1223 plasmid prior to transformation.
Remark: Sequencing revealed the unexpected insertion of an additional gypsy element 5' of the *PUb* indicated by an additional ">".

d) pET SUMO-GoldenGate and pre-toxin expression vectors

pET SUMO-GoldenGate **- (KNE001)**

The generation of KNE001 *pET SUMO-GoldenGate* was based on *pET SUMOadapt* (Bosse-Doenecke et al., 2008) which is a modified version of the *pET SUMO* expression vector (Hanington et al., 2006; Mossessova and Lima, 2000) (MTA with Cornell University, U.S.A.; available at *Invitrogen^{TM}/ Thermo Fisher Scientific*, Waltham, U.S.A.) extended by a MSC-adapter sequences. This adapter comprises, among other typical type II restriction sites, a *Bsa*I type IIS recognition site, enabling residue-free and in-frame cloning of the CDS of interest with the *ATG::6xHis::SUMO* ORF. To create *pET SUMO-GoldenGate* a second *Bsa*I site was integrated along with the *CAT* resistance and *ccdB* death cassette (Bernard et al., 1994; Engler et al., 2008). With appropriate design of *Bsa*I cleavage sites (≠ recognition site) in the GoldenGate-linker sequences that are added as 3'-overhangs to the gene-specific forward and reverse primer, respectively, *pET SUMO-GoldenGate* and the PCR amplified CDS and be cut and ligated

into a product lacking the original restriction sites. The *ccdB* cassette in parental KNE001 ensures that only positive colonies can grow. Unless the CDS of the GOI contains a *Bsa*I restriction site, the following GoldenGate linker sequences can be deployed: for forwards primer *CCAGGTCTCATGGT*; for reverse primer *GGGGGTCTCCTCGAG*.

(i) At first, a 1.7 kb fragment containing the *lac* promoter, the *CAT* gene and the *ccdB* death cassette was PCR amplified with primers *GG_ccdB_F/ GG_ccdB_R* (Table 3.2.4 A) from *pTALEN(NI)v2* (Sanjana et al., 2012). *pTALEN_v2 (NI)* was a gift from Feng Zhang (Addgene plasmid # 32189). The overhang of the reverse primer *GG_ccdB_R* added a *Xho*I-site to the 3' of the amplicon.

(ii) Subsequent *Not*I/*Xho*I digestion of the amplicon resulted in a 1.5 kb *Not*I_*lacP-CAT_ccdB_Xho*I fragment which was ligated in the *Not*I/*Xho*I linearized *pET SUMOadapt*. *pET SUMO-GoldenGate* was transformed and propagated in ccdB Survival™ 2 T1R Competent Cells (*Thermo Fisher Scientific*, Waltham, U.S.A.). (Eckermann, 2013).

***pET SUMO-Cry4Aa* - (KNE002)**

Cloning of the pre-toxin CDS of *Crystal4A* in *pET SUMO-GoldenGate* to create KNE002 *pET SUMO-Cry4Aa* was done through the following steps (Eckermann, 2013):

(i) The DNA fragment containing the *Cry4Aa* CDS was generated via PCR, using the primers *GG_Cry4A_fMet_F/ GG_Cry4A_FL_R* and the plasmid *pMEx-B4A* (Boonserm et al., 2004) as template. The primers added the specific GoldenGate linker sequence to the 5' and 3' end of the amplicon, respectively. *pMEx-B4A* was a kind gift from Chanan Angsuthanasombat (Mahidol Universitym Thailand).

(ii) The performed GoldenGate reaction was modified from the previously described procedure (Engler et al., 2008). To this end, the reaction was done in a total volume of 10 µl containing 100 ng of the *pET SUMO-GoldenGate* vector, the *Cry4Aa* CDS comprising fragment generated in (i) (1:10 molar ratio, vector: insert), 0.75 µl T7 *Bsa*I HF, 1 µl NEB Cutsmart buffer, 1 µl 1X BSA, 1 µl of ATP [10 mM] and 0.25 µl T7 ligase. The reaction took place during 40 cycles alternating 10 min at 37°C and 10 min at 20°C with subsequent heat-inactivation for 20 min at 70°C.

***pET SUMO-MNL* - (KNE026)**

Cloning of the pre-toxin CDS of *Monalysin* in *pET SUMO-GoldenGate* to create KNE026 *pET SUMO-MNL* was done through the following steps:

(i) The DNA fragment containing the *MNL* CDS was generated via PCR, using the primers *GG_Linker_MNL_F/ GG_Linker_MNL_R* and the plasmid pETG-20A-*Monalysin* (Opota et al., 2011) as template. The primers added the specific GoldenGate linker sequence to the 5' and 3' end of the amplicon, respectively. pETG-20A-*Monalysin* was a kind gift from Bruno Lemaitre (Ecole Polytechnique Fédérale Lausanne) and Alain Roussel (Architecture et function des macromolecules biologiques, Marseille).

(ii) The performed GoldenGate reaction was modified from the previously described procedure (Engler et al., 2008). To this end, the reaction was done in a total volume of 10 µl containing 100 ng of the *pET SUMO-GoldenGate* vector, the *MNL*CDS comprising fragment generated in (i) (1:10 molar ratio, vector: insert), 0.75 µl T7 *Bsa*I HF, 1 µl NEB Cutsmart buffer, 1 µl 1X BSA, 1 µl of ATP [10 mM] and 0.25 µl T7 ligase. The reaction took place during 40 cycles alternating 10 min at 37°C and 10 min at 20°C with subsequent heat-inactivation for 20 min at 70°C.

Table 3.2.4 A: Oligonucleotides

Primer name	*Sequence*
DP99AgeIF	*AGCATACCGGTCGTCAAGGTCTAAAGGGAGCA*
DP99NheIR	*AGCATGCTAGCGACGGATTGGCAACGATTGTG*
IC102	*GCGGAGACTCTAGCGGAAGTACACTCTTCATGGCGATA*
IC30	*TGCTAGAGTTTTCATGGTGTCTCTGGATTAGACGACT*
IC86	*ATGGTGAGCAAAGGCGAAGAAG*
IC91	*CTTCACAAAGATCCTCTAGATTATTTATACAGTTCAT CCATGCCG*
IC10	*ATGAAAACTCTAGCACTATT*
IC93	*TCTCCTTTACTCATGGATCCACATCTTCCACC CCAGGCGG*
IF_TRE_F	*CCGAATTCTGCAGATGGCGCGCCTAGGGTGCC*
IF_TRE_R	*CGATATCAAGCTTATCGATGG*
IF_SP::MNL_F	*ATAAGCTTGATATCGATGAAAACTCTAGCACTATTCTTGG*
IF_SP::MNL_R	*ACATCTTCCACCCCAGGCGG*
IF_MNL_C1_F	*TGGGGTGGAAGATGTACGATCAAGGAAGAGCTGG*
IF_MNL_pCRII_R	*GCCAGTGTGATGGATTCAATAACGGCGATGAGG*
IF_MNL_R	*TCAATAACGGCGATGAGGATCGTTGTAGG*
IF_SV40_F	*CATCGCCGTTATTGAGAATTCCTGCAGCCCGGG*
IF_SV40_AscI_R	*GCCAGTGTGATGGAT GGCGCGCCAAGCTTGGT*
Gib_SP:MNL_Ins_F	*TGCCATACCATTTAGCTAGGCCGGCCTTGGCGCGCCTAGGGTGCCCCA*
Gib_SP:MNL_Ins_R	*TATGCAATGTTTTTGCGAATAGGGTACCGGCGCGCCAAGCTTGGTCGAG*
GG_ccdB_F	*ACATGATTGCGGCGTTGCC*
GG_ccdB_R	*TGTCTCTCGAGGAGACCGTCGACCTGCAGACT*
GG_Cry4A_fMet_F	*CCAGGTCTCATGGTATGAATCCTTATCAAAATAAAAATG*
GG_Cry4A_FL_R	*GGGGGTCTCCTCGAGTCACTCGTTCATCCAAAT*
GG_Linker_MNL_F	*CCAGGTCTCATGGTATGACGATCAAGGAAGAGCTGG*
GG_Linker_MNL_R	*GGGGGTCTCCTCGAGTCAATAACGGCGATGAGG*

3.2.4.2.4 Germline transformation, tissue dissection, transient transfection of Schneider 2 cells, sterility assay and toxicity assay

Germline transformation

Germline transformation, DNA preparation, backcrossing and screening for transgenic *D. melanogaster* and *C. capitata* flies was performed using the helper plasmid *Dm-^{m}hyPBase* as described in *chapter 3.1* (Eckermann et al., 2018) based on the previously described procedures (Handler et al., 1998; Spradling and Rubin, 1982), including the documented modifications.

Dissection and imaging of *Drosophila melanogaster* male reproductive tract

Sexually mature males were anesthetized with CO_2 and decapitated. Dissection and imaging of the male reproduction tract was performed in 1X PBS under a Leica M205 FA fluorescent stereo microscope (*Leica Microsystems GmbH*, Wetzlar, Germany) with the corresponding filters.

Transient transfection of Schneider 2 cells

Firstly, the cell density was determined with a Neubauer counting chamber. Fresh S2 cell culture medium (see 0) was added to the cell suspension to obtain a dilution of 1 x 10^6 cells/ ml. For transient transfection 2 x 10^6 cells in a total volume of 2 ml were placed in a well of a 6-well plate (*Corning Incorporated*, New York, U.S.A.) the day before transfection. Then cells were co-transfected with 1 µg of either the *pJFRC7[20XUAS_Ac5c-mCherry]* (KNE009) or the *pJFRC7[20XUAS_Ac5c-SP::mCherry]* (KNE009) plasmid together with 1 µg of the *p[Ac5C-Gal4]* helper plasmid (kindly provided by Andreas Wodarz, University of Cologne). The final transfection mix contained a total amount of 2 µg of plasmid DNA and 4 µl of FuGENE® 6 (*Promega*, Madison, U.S.A.) lipofectamine filled up with ddH_2O to a total volume of 100 µl per tested construct. After 48-72 hours of incubation, the medium was replaced by fresh S2 cell culture medium and S2 cells were used for protein assays or immunohistochemistry. (Eckermann, 2013).

Sterility Assay

To examine for potential male sterility effects that result from a combination of the *Ccβ2t-tTA* driver and the *TREhs43-hid*^{Ala5} effector constructs, we crossed female *C. capitata* virgins of each of the four homozygotized *Ccβ2t-tTA* driver lines DF6-P1, DF6-P1, DF14 and DF25 (Eckermann et al., 2018) to *C. capitata* males of the homozygous *TREhs43-hid*^{Ala5} effector line (F1m2) (Schetelig et al., 2009a), to generate double-heterozygous killed-sperm flies (KiSp#1, KiSp#2, KiSp#3, and KiSp#4, repectively) carrying both, the driver and effector construct. Subsequently, virgin siblings from each of the four double-heterozygous KiSp "lines" were inbred. Eggs originating from these crosses were collected and larval hatch-rates were examined under restrictive conditions (without tetracycline) in three replicates á 100 embryos. Furthermore, additional eggs from the same crosses were collected and given onto a permissive larval diet containing 10 µg/ml tetracycline to generate double-homozygous KiSp lines (KiSp#1, KiSp#2, KiSp#3, and KiSp#4) and prevent a potential premature activation of the killed-sperm system during the process of inbreeding. Identification and selection of *C. capitata* flies carrying the driver and/or effector construct in hetero- or homozygous state was done based on fluorescence signal intensity of the construct-specific marker under a Leica M205 FA fluorescent stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany). Selected double-homozygous opposite sex virgin siblings of the respective KiSp lines were then crossed and the larval hatch-rates of collected eggs was examined in three replicates á 100 embryos for each of the lines.

Cry4A Toxicity Assays

Toxicity assay of recombinant Cry4A pre-toxin to *Ae. Aegypti* larvae

The assay was performed in three replicates, each by placing 15 first or second instar larvae into 7.5 ml of rearing water (Hays and Raikhel, 1990; Lea, 1964), which contained a pro-Cry4A protein contraction of either 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml or 25 µg/ml (Cry4A stock solution: 0.7 mg/ml in 150 mM NaCl, 2 mM KCl, 2 mM, $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM HEPES, pH 7.5 Ringer's solution). For the control experiments the corresponding volumes of only Ringer's solution were added instead. The toxicity was evaluated by monitoring dead larvae after 24 h, 48 h, 72 h and mosquitos that have reached adulthood.

Feeding toxicity assays of recombinant Cry4A pre-toxin to *D. melanogaster* and *C. capitata* larvae

The *D. melanogaster* diet and *C. capitata* larval diet containing the recombinant Cry4A pro-toxin were prepared based on the description for standard relaxed artificial rearing conditions by Roberts (1998) and Saul (1982), respectively. The assays were performed in three replicates, each by placing 25 first instar

larvae onto 5 ml of the species-specific diet, which contained a Cry4A protein contraction of either 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, or 25 µg/ml (Cry4A stock solution: 0.7 mg/ml in 150 mM NaCl, 2 mM KCl, 2 mM, $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM HEPES, pH 7.5 Ringer's solution). For the control experiments the corresponding volumes of only Ringer's solution were added instead. The toxicity was evaluated by counting the number of pupae and eclosed adult flies.

3.2.4.2.5 Protein biochemistry

Protein isolation from Schneider 2 cells

Total protein-extraction from a transient transfected *D. melanogaster* Schneider 2 cell culture was done based on the principles of the previously described protocol (Wodarz, 2008). After removal of the S2 cell culture medium, cells were resuspended in phosphate-buffered saline (PBS, pH 7.0). The cell suspension was briefly centrifuged (RT, 3.000 rcf) and the cell pellet was resolved in 200 µl lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 % Triton X-100), including protease inhibitor cocktail (*Roche Diagnostics GmbH,* Mannheim, Germany). For cell lysis, cells were placed on ice for 15 min, shaking, with subsequent centrifugation (4 °C, 14.000 rcf) for 15 min. The protein concentrations of the supernatant was measured photometrical with Bradford and was then mixed with 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol). (Eckermann, 2013).

Expression and purification of recombinant pre-toxins

The expression and purification of recombinant pre-toxins was conducted as previously described (Monecke et al., 2014), with the described individual modifications for Cry4A and MNL, respectively.

Expression and purification of recombinant Cry4Aa pre-toxin

Escherichia coli BL21 star (DE3) chemically competent cells (*Thermo Fisher Scientific*, Waltham, U.S.A.) were transformed with the *pET SUMO::Cry4Aa* (KNE002) expression vector. Two liter of kanamycin-containing phosphate buffered TB-medium were inoculated with an o.n. culture to an initial OD_{600} of 0.1 and incubated at 37°C until cell density reached an OD_{600} of 0.8 when expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM. The culture was then shifted to 16°C. 18 h after induction with IPTG *E. coli* cells were harvested by centrifugation at 5000 rcf and 4°C for 20 min. The obtained cell pellet was resuspended in 5-10 ml lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 2 mM β-mercaptoethanol, 10 mM imidazole) per 1 g cell pellet.

Cell disruption was done mechanically utilizing a M-110S Microfluidizer® (*Microfluidics Corp.*, Westwood, U.S.A.). To separate soluble from insoluble cell content, the lysate was centrifuged at 30,000 rcf and 4°C for 30 min and the resulting supernatant was taken for subsequent immobilized metal ion affinity chromatography. To this end, the supernatant was injected onto two stacked and previously with lysis buffer equilibrated Protino® Ni-NTA Columns (5 ml) (*MACHEREY-NAGEL GmbH & Co. KG*,

Düren, Germany), which were installed to an ÄKTAprime (*GE Healthcare Europe GmbH*, Freiburg, Germany). Prior to elution with a linear gradient of the elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 2 mM β-mercaptoethanol, 500 mM imidazole) the columns were washed with two bed volumes of lysis buffer.

Samples of the collected fractions were examined for presence of the (6x)His::SUMO::Cry4Aa fusion-protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent coomassie brilliant blue staining (see below: SDS-PAGE). Fractions containing the fusion-protein were pooled and given together with SUMO protease (1:100 molar ratio, protease: fusion-protein) (kindly provided by Achim Dickmanns, University of Göttingen) into a Spectra Por®1 Dialysis Tubing (6-8 kDa MWCO) (*Spectrum Europe B.V.*, Breda, Netherlands), which was placed o.n. at 4°C into a bath of desalting buffer (300 mM NaCl, 10 mM NaH_2PO_4) to remove the imidazole.

The protein solution was then concentrated using an Amicon® Ultra-15 Centrifugal Filter (Ultracel® - 50K) (*Merck Millipore Ltd.*, Cork, Ireland) to a total volume of 13 ml which was loaded onto a HiLoad 26/600 Superdex 200 pg (*GE Healthcare Europe GmbH*, Freiburg, Germany) gel filtration column for the last step of Cry4Aa purification by size-exclusion chromatography with a modified Ringer's solution (150 mM NaCl, 2 mM KCl, 2 mM, $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM HEPES pH 7.5) as storage buffer. The collected fractions were again examined for Cry4Aa content, then pooled, concentrated (see above), aliquoted, and frozen in liquid nitrogen for storage at -80°C.

Expression and purification of recombinant MNL pre-toxin

Expression and purification of recombinant MNL protein was performed in analogy to the above described procedure for expression and purification of Cry4Aa with the below listed differences.

(i) *Escherichia coli BL21 (DE3)pLysS* chemically competent cells (*Promega*, Madison, U.S.A.) were transformed with the *pET SUMO::MNL* (KNE026) expression vector.

(ii) For induction of expression, IPTG was added to a final concentration of 0.7 mM.

(iii) Lysis buffer (500 mM NaCl, 50 mM Tris-HCl, 10 mM imidazole, pH 8), elution buffer (500 mM NaCl, 50 mM Tris-HCl, 250 mM imidazole, pH 8) and desalting buffer (500 mM NaCl, 10 mM Tris-HCl) were modified from Opota et al. (2014). 1X PBS was used as storage buffer.

(iv) Concentration of protein solutions prior to and after gel filtration was done with Amicon® Ultra-15 Centrifugal Filter (Ultracel® - 10K) (*Merck Millipore Ltd.*, Cork, Ireland) to a total volume of 5 ml.

(v) For size-exclusion chromatography a Hi Load 16/600 Superdex 75 pg (*GE Healthcare Europe GmbH*, Freiburg, Germany) gel filtration column was used.

Remark: The immobilized metal ion affinity chromatography resulted in two distinct absorption peaks (at 280 nm), which revealed upon examination of the respective fractions that MNL was eluted specifically at two different imidazole concentrations. Therefore, separate gel filtrations were done for pooled fractions of the respective peaks.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) & Western blotting

SDS-PAGE and Western blotting was performed as previously described (Wodarz, 2008). Information regarding the separation range (kDa) of polyacrylamide gels and therefore the different compositions with respect to the acrylamide percentage were taken from the "*Info Brochure PAGE Instructions"* (*Carl Roth GmbH + Co. KG*, Karlsruhe, Germany). Protein bands in SDS-polyacrylamide gels were visualized with Coomassie Brilliant Blue. To this end, gels were briefly heated in a Coomassie staining solution consisting of 50 ml staining/de-staining solution (10% EtOH, 5% acetic acid) and 2 ml Coomassie solution (0.75% G-250, 0.25% R-250, in EtOH) and were further incubated, until protein bands were visible. To remove unspecific staining, gels were subsequently heated and incubated in 50 ml staining/de-staining solution. Incubation for staining and de-staining was done on a gently moving rocking platform. Antibodies used for Western blotting in this study can be reviewed in Table 3.2.4 B.

Table 3.2.4 B Antibodies used for Western blotting

	antibody	*working dilution*	*species*	*manufacturer*
primary antibodies	Anti-RFP (PM005)	1:500	rabbit	*MBL International Corporation*, Woburn, U.S.A.
	His•Tag® Monoclonal Antibody (70796)	1:500	mouse	*Merck Millipore*, Billerica, U.S.A.
secondary antibodies	anti-Rabbit IgG (H+L)-HRPO (111-035-144)	1:10000	goat	*Dianova GmbH*, Hamburg, Germany
	anti-Mouse IgG+IgM (H+L)-HRPO (115-035-068)	1:10000	goat	*Dianova GmbH*, Hamburg, Germany

3.2.4.2.6 Immunohistochemistry

Immunohistochemistry of *D. melanogaster* Schneider 2 cells

Following transfection, cells were resuspended and a coverslip was given into the well. After the cells have settled and adhered to the coverslip, the culture medium was removed and cells were gently washed with 1x PBS prior to fixation with 4% paraformaldehyde in PBS for 15 min, which was followed again by three washing steps with PBS for 10 min each. Subsequent blocking was done with 5% normal horse serum (NHS) in PBT (PBS with 0.1% Tween-20) for 30 min. Incubation of the fixed cells with the primary polyclonal rabbit Anti-RFP antibody (1:2000) (PM005, *MBL International Corporation*, Woburn, U.S.A.) and the secondary antibody Alexa Flour®647 conjugated goat anti-rabbit (1:200) (A-21245*, Invitrogen/ Thermo Fisher Scientific*, Waltham, U.S.A.) was done simultaneously in PBT including 5% NHS for 2 h at RT. Following three washes with PBS, cells were stained with DAPI (1:1000 in PBT) (*Molecular Probes™/ Thermo Fisher Scientific*, Waltham, U.S.A.) for 10 min at RT. After the two last washes with PBS, coverslips with stained cells were mounted upside down on an objective slide with Mowiol®4-88 (*Sigma-Aldrich*, Missouri, U.S.A.) embedding medium. Immunocytochemical cells were imaged with a LSM 510 META Confocal Laser Scanning Microscope (*Carl Zeiss Microscopy GmbH*, Jena, Germany). (Eckermann, 2013).

3.3 Consequences of resistance evolution in a Cas9-based sex-conversion suppression gene drive for insect pest management

In this chapter, we evaluate the use of the novel genomic editing tool CRISPR/Cas9 to develop gene drive systems as a new pest control strategy. We present detailed insights into the frequency and the underlaying mechanisms of target site resistance formation in a "simple" homing-based gene drive design. Our findings demonstrate that targeting essential genes, such as *transformer* in *Drosophila melanogaster*, creates a hotspot for resistance allele development due to the system-imposed high selective pressure. To overcome this issue, we proposed the use of multiple guide RNAs for future gene drive systems. While targeting *transformer* in *D. melanogaster* leads to sterile XX intersexes, destruction of the same gene in the agricultural pest *C. capitata* results in fertile XX males. With this in mind, and based on our obtained data, we developed a model, which predicts successful suppression of *C. capitata* populations, if the suggested modifications are implemented.

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My specific contributions were particularly to Fig. S4, Fig. S6, Fig. S7, and Fig. S8, leading to the main Fig. 3. Moreover, I designed and prepared all figures, except for Fig. S9, Fig. S10, Fig. S11, and partially main Fig. 4.

Consequences of resistance evolution in a Cas9-based sex-conversion suppression gene drive for insect pest management

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The use of a site-specific homing-based gene drive for insect pest control has long been discussed, but the easy design of such systems has become possible only with the recent establishment of CRISPR/Cas9 technology. In this respect, novel targets for insect pest management are provided by new discoveries regarding sex determination. Here, we present a model for a suppression gene drive designed to cause an all-male population collapse in an agricultural pest insect. To evaluate the molecular details of such a sex-conversion–based suppression gene drive experimentally, we implemented this strategy in *Drosophila melanogaster* to serve as a safe model organism. We generated a Cas9-based homing gene-drive element targeting the *transformer* gene and showed its high efficiency for sex conversion from females to males. However, nonhomologous end joining increased the rate of mutagenesis at the target site, which resulted in the emergence of drive-resistant alleles and therefore curbed the gene drive. This confirms previous studies that simple homing CRISPR/Cas9 gene-drive designs will be ineffective. Nevertheless, by performing population dynamics simulations using the parameters we obtained in *D. melanogaster* and by adjusting the model for the agricultural pest *Ceratitis capitata*, we were able to identify adequate modifications that could be successfully applied for the management of wild Mediterranean fruit fly populations using our proposed sex-conversion–based suppression gene-drive strategy.

homing endonuclease | integrated pest management | molecular entomology | sex reversal | Tephritid fruit flies

The use of CRISPR-Cas9 systems as a homing-based gene-drive tool to alter the genotype of insect populations has theoretically (1–5) and practically (6–8) been shown to be feasible. These systems can potentially allow the spread of any desired trait in a wild population of target species even if the desired phenotype imposes a fitness cost (2, 4, 5, 8). Therefore, the spread of lethality or sterility traits that could result in suppression and eventually collapse of the target population should be possible. This has recently attracted special attention in pest and disease vector control (1, 3, 6–8). However, the effort had focused mainly on disease-vector mosquitoes such as *Anopheles* (7, 8). In homing CRISPR/Cas9 gene-drive (HCGD) systems, a CRISPR/Cas9 homing element (CHE) composed of at least the Cas9 endonuclease-coding sequence and a guide RNA (gRNA) is integrated in the host genome at the gRNA target site. In the heterozygous state, Cas9 introduces an RNA-guided double-strand break in the wild-type allele (similar to homing endonucleases) which then will be repaired either by homology-directed repair (HDR) or error-prone mechanisms such as nonhomologous end joining (NHEJ). In the former case, the CHE allele serves as the repair template and is copied into the homologous chromosome. Directing this process to the germline will result in super-Mendelian inheritance driving the CHE and any accompanying genes into the population. Therefore, the highly customizable nature of CRISPR/Cas9 allows simple design of HCGDs to drive any desired trait, even those resulting in sterility, into wild populations as long as the cost of this phenotype does not surpass a certain threshold (1, 4).

In a recent study, Hammond et al. (8) identified a set of genes whose knockout resulted in female-specific sterility in *Anopheles*. However, they found that only one of these genes could be used as a target for HCGDs to achieve an efficient drive of female-specific sterility into the population. The remaining sterility genes imposed a very strong cost on the carriers that eventually resulted in the elimination of the drive allele from the population. As predicted by mathematical population genetics models, the spread of female-specific sterility traits in a population using HCGDs should eventually result in a population collapse and local or global elimination of the target species (1, 8). Another proposed strategy to achieve this goal is to design drive elements that alter the population's sex ratio toward males. Surprisingly, such gene-drive elements have naturally been observed in some organisms. In *Aedes aegypti*, for example, a type of drive element

Significance

Resistance evolution caused by CRISPR/Cas9 gene-drive systems has a major impact on both the future scientific design of such gene-drive systems and on the politics of regulating experimentation and use of such systems. In our study, we show that in-frame drive-resistant alleles can be produced readily and inherently in a suppression gene-drive system. The rate at which such alleles emerge will determine the maximum size of the population that could be targeted for collapse and elimination. Here, we provide a potential target site and the modeling framework for implementation and optimization of a suppression gene-drive strategy to control Mediterranean fruit fly populations.

Author contributions: M.K., H.M.S.C., S.D., J.M.M., and E.A.W. designed research; M.K., K.N.E., H.M.M.A., H.M.S.C., and J.M.M. performed research; M.K., K.N.E., H.M.M.A., H.M.S.C., S.D., J.M.M., and E.A.W. analyzed data; M.K., K.N.E., J.M.M., and E.A.W. wrote the paper; and H.M.S.C. and J.M.M. designed and analyzed the population dynamic models.

The authors declare no conflict of interest.

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known as a "Killer-Y chromosome" is able to shatter the X chromosome during spermatogenesis, and therefore all offspring of mosquitoes carrying such a chromosome will be male. To replicate this phenomenon, Galizi et al. (9) employed a specific homing endonuclease, I-Ppol, to specifically shatter the X chromosome during spermatogenesis of *Anopheles gambiae*. By generating transgenic males carrying an engineered version of such a homing endonuclease gene (HEG) on somatic chromosomes, they have shown that at high initial load frequencies these flies will result in population collapse in cage experiments. They proposed that integration of such a HEG on the Y chromosome could be an effective gene-drive strategy for population control of *An. gambiae*. The distortion of the sex ratio using an X chromosome-specific CRISPR/Cas9 system has also been shown to be successful in *An. gambiae* (10).

Here, we propose an independent approach that converts female individuals into fertile males by disturbing the developmental sex-determination pathways, which distorts the sex ratio without adverse effects on the reproductive success of carrier males. A prime target gene to achieve this goal is *transformer* (*tra*). *tra* plays a pivotal role in female sex determination in different insect orders, including Diptera (11). In a devastating agricultural fruit pest, the Mediterranean fruit fly, *Ceratitis capitata* ("medfly"), *tra*-knockdown XY males develop normally, while XX individuals develop as fertile males (12). Therefore, *C. capitata* XX males carrying a CHE-targeted *tra* locus could further spread the CHE to all their progeny (Fig. 1*A*), resulting in an effective gene drive without any direct effect on the fecundity of individuals carrying the drive element. This in theory could lead to an all-male population collapse that can be used for controlling the wild population of this aggressive pest.

Because of the strict guidelines on gene-drive experiments and to adhere to recommendations of scientific communities (13–15), we decided to test this gene-drive strategy first using *Drosophila melanogaster* as a model organism. In *D. melanogaster*, *tra*-mutant XX individuals develop into infertile pseudomales (16), not giving rise to further progeny (Fig. 1*B*). Since the cost of this infertility is significantly higher than the threshold tolerated by gene-drive systems (1, 4), a CHE targeting the *tra* locus in *D. melanogaster*, despite its ability to show super-Mendelian inheritance in individual crosses, is not able to drive into a population (Fig. 1 *C* and *D*). This biological confinement allows us to employ *D. melanogaster* as a safe model organism for studying the limitations of our suggested suppression gene-drive systems at the molecular level in the laboratory and thereby experimentally identify parameters that might need to be adjusted to achieve an efficient suppression gene-drive system in *C. capitata*.

In our study, we found that targeting *tra* works as an efficient means of sex conversion in *D. melanogaster*. However, the early onset of the formation of in-frame drive-resistant alleles compromises drive efficiency. Based on our observations, we simulated the use of a *tra*-based suppression gene-drive system for control of *C. capitata* populations and showed that HCGD systems employing multiple gRNAs that target the *tra* locus can serve as an effective pest-control strategy for *C. capitata*.

Results

Design of a *tra*-Based Sex-Conversion Suppression Gene-Drive System. The proposed CHE is composed of a *spCas9*-coding sequence under the control of a suitable promoter, as explained below, a gRNA targeting the first exon of *tra* under the control of a *Pol III* promoter, and a fluorescent marker to identify the genomic integration (*SI Appendix*, Fig. S1*C*). The activity of this CHE unit will be similar to that of homing endonucleases and would be able to perform homing into the wild-type *tra* allele. For our *tra*-targeting CHE to drive in a population, it is essential that Cas9 is expressed in the germ cells to promote homing into the wild-type *tra* allele by HDR. To achieve sex conversion, however, *tra* needs to be inactivated in the somatic cells of XX individuals. Thus two

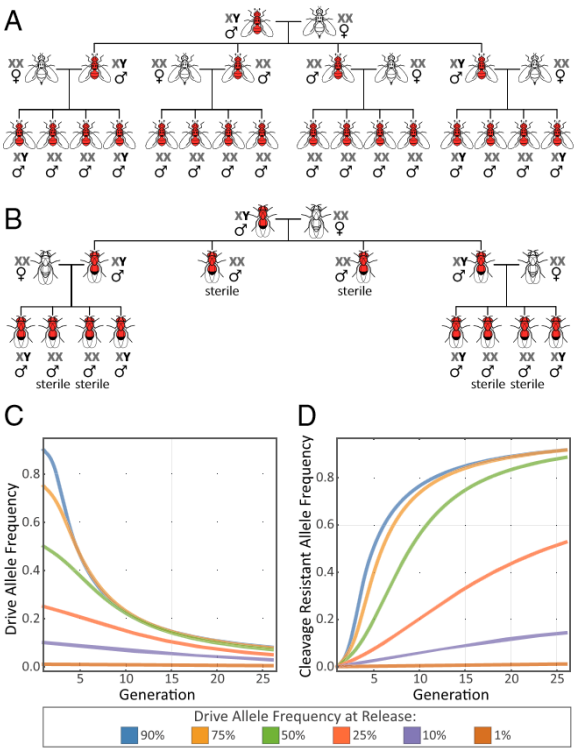

Fig. 1. Insect suppression gene drive based on forced all-male offspring. (*A*) In *C. capitata*, a *tra*-targeting CHE with both germline and somatic expression causes super-Mendelian inheritance of the red fluorescence-marked CHE-null allele but also results in the transformation of XX individuals into males and in theory leads to a subsequent collapse of the population. (*B*) In *D. melanogaster*, homing into *tra* in somatic cells transforms XX individuals into sterile pseudomales, which halts the spread of the selfish element but allowed us to safely study the dynamics and molecular consequences of using CHEs in a suppression gene-drive system. (*C*) Predicted transience of a Cas9-based homing construct targeting the *tra* locus in *D. melanogaster*. Predictions are based on the introduction of tra^{nCHE}/+ males at frequencies of 1–90% into a population of otherwise wild-type males and females in equal proportion. We assume a Cas9-mediated cleavage efficiency of 100%, a probability of accurate HDR following cleavage of 90%, one-third of drive-resistant alleles (NHEJ products) being in-frame indels, and no fitness cost associated with in-frame drive-resistant alleles. A construct having these parameter values and released in the form of tra^{nCHE}/+; XY males is expected to be reduced to an allele frequency of less than 10% within ~25 generations with a trajectory tending toward elimination regardless of the introduction frequency. (*D*) At high release frequencies, the presence of the drive allele results in the generation and establishment of cleavage-resistant alleles in the population. At low release frequencies, which may occur because of accidental escapes, the drive allele will be eliminated early, and the cleavage-resistance alleles will appear at only negligible frequencies. This indicates that *D. melanogaster* is a safe model organism for the evaluation of a *tra*-based suppression gene drive causing sex conversion.

scenarios in XX individuals heterozygous for the drive allele are plausible: (*i*) Cas9 protein is expressed only in a fraction of the cells, and its activity results in the development of mosaic intersex individuals or (*ii*) Cas9 is expressed in all somatic cells and uniformly destroys the wild-type *tra* allele, resulting in the development of XX males. In *C. capitata*, the latter will result in development of fertile XX males (12), which can further spread the drive allele into the population (Fig. 1*A*). It is important to note that it is irrelevant whether the mutation of the wild-type *tra* allele in the somatic cells is based on HDR or NHEJ as long

as the mutation disrupts the function of *tra* and thereby causes sex conversion.

Therefore, the combination of germline homing at the *tra* locus (which results in the spread of the drive allele) and somatic targeting of the wild-type *tra* allele (which results in sex conversion) is needed to enable our proposed suppression gene-drive strategy to be effective. To achieve this, different types of promoters or combinations thereof could be used. (*i*) A germline-specific promoter could be combined with an early zygotic promoter from a cellularization gene for high and ubiquitous blastoderm expression (17). Such cellularization promoters have already been successfully applied for transgenic approaches in *C. capitata* (18). It is important to note that these early cellularization genes are not expressed in the primordial germ cell (PGC) nuclei (19, 20), which are therefore not exposed to NHEJ-based mutation in the early embryo (21, 22). In *D. melanogaster*, one of these cellularization genes, *Sry-α*, is in fact expressed both in a somatically limited way in the blastoderm and in the PGCs at later developmental stages (23, 24), and its promoter therefore might be sufficient for both germline homing and somatic sex conversion. (*ii*) Since Pol II-dependent transcription is actively suppressed in the PGCs (25), a ubiquitous cell cycle-specific promoter, such as the *DNApol-α180* promoter (26), could result in uniform targeting of all cells during development except early-stage PGCs. The paternal-only transmission of our proposed gene-drive strategy is likely to help overcome the problem of DNA cleavage at early embryonic stages when HDR is unlikely to occur (21, 22) and therefore is expected to result in both uniform sex conversion and germline homing. (*iii*) Since the target gene *tra* is expressed in the somatic cells at very early embryonic stages, the genomic context might mediate suitable amounts of expression independently of the introduced promoter. Thus, the introduction of a germline-specific promoter, such as the *Rcd-1r* promoter, which had previously been shown to result in efficient homing-based gene drive in *D. melanogaster* (27), might by itself be sufficient to drive Cas9 expression for both purposes.

***D. melanogaster* as a Safe Model System for Evaluation of a *tra*-Based Suppression Gene Drive.** In our experiments, we followed the recommended physical containment procedures (15) (*SI Appendix, SI Materials and Methods*). Moreover, since in *D. melanogaster* XX males are always sterile, the somatic sex conversion imposes a strong fitness cost on the XX individuals carrying the drive allele, which impedes the spread of the drive allele in the population (1, 4), rendering *D. melanogaster* a safe model system to study this suppression gene-drive strategy at the molecular level (Fig. 1*B*). Nevertheless, to ensure that the use of a CHE against the *tra* locus in *D. melanogaster* is indeed biologically confined in case of an unlikely accidental escape, a deterministic model for an ideal scenario (homing efficiency of 90% and assuming that one-third of NHEJ events result in the formation of in-frame indels) based on predicted phenotypic outcomes of the drive in *D. melanogaster* was used. The modeling graphs demonstrate that, because of its high fitness cost, even at 90% initial frequency a CHE targeting the *tra* locus not only is unable to drive into a population but also is actively eliminated from the population (Fig. 1*C*). In this example, the presence of the drive allele at high frequencies may result in the generation of cleavage-resistant alleles, which theoretically could alter the genetic makeup of the population at the targeted locus (28). However, our results indicate that at the low release frequencies (<1%) that are expected in case of an accidental release, the drive allele becomes eliminated from the population at very early stages without any significant effect on the wild population (Fig. 1*D*). Therefore, it is safe to assume that such a drive system is biologically confined in *D. melanogaster* and thus meets the recommendations for gene-drive experiments (13–15, 28).

Implementation of the *tra*-Based Suppression Gene-Drive System in *D. melanogaster*. Since our sex-conversion suppression gene-drive system requires both somatic and germline Cas9 activity, we tested three different promoters (*SI Appendix*, Fig. S1): (*i*) the *Sry-α* promoter, (*ii*) the *DNApol-α180* promoter, and (*iii*) the *Rcd-1r* promoter. We also included the 3′ UTR of the *β2 Tubulin* (*βTub85D*) gene at the 3′ end of the *Cas9* transcript, as it had been shown to increase the homing efficiency in *D. melanogaster* (27). Moreover, the first intron of *αTub84B* was inserted upstream of the Cas9 coding sequence to further enhance *Cas9* expression (29).

To allow the simple generation of various strains in an isogenic background for these promoters, we used a transgenesis approach similar to that demonstrated in *Anopheles* (*SI Appendix*, Fig. S2) (8). First, a tra^{nDOCK} strain was established by site-specific integration of a recombinase-mediated cassette exchange (RMCE) docking site into the first exon of the *tra* gene using an efficient gRNA (*SI Appendix*, Fig. S1 *A* and *B*). Second, to generate the homing strains for each of the promoters, RMCE was performed in tra^{nDOCK} embryos using ϕC31 integrase. All individuals that carried the CHE allele (tra^{nCHE}) were found either to be males or to show a mosaic intersex phenotype, indicating that targeting the *tra* locus is indeed an efficient sex-conversion strategy in *D. melanogaster* (*SI Appendix*, Fig. S3*A*).

To assess the efficiency of each promoter in performing gene drive as well as inducing somatic sex conversion, 10 males from each of the tra^{nCHE} strains and the tra^{nDOCK} strain were individually crossed with w^- virgins, and the ratio of females in the F_1 generation from each single cross was determined (*SI Appendix*, Fig. S1*D*). The results show that all three promoters can block female development of heterozygous (tra^{nCHE}/+) XX individuals (somatic sex conversion) and drive into the next generation (germline activity). However, since HDR in the germline is of key importance for the molecular study of gene drive, we continued our experiments with the *Rcd-1r* strain.

To evaluate the drive efficiency of this CHE and the rate at which the *tra* locus is targeted to cause sex conversion, 12 heterozygous (tra^{nCHE}/+) males were crossed individually with virgin w^- flies. Screening the F1 progeny revealed that up to 92% of the individuals carried the DsRed eye marker (on average 78%, corresponding to a homing efficiency of 56%), and up to 96% (on average 89%) were males/intersexes (*SI Appendix*, Fig. S3 *B* and *C*). These results further confirmed that our proposed suppression gene-drive strategy is indeed able to perform super-Mendelian inheritance, similar to findings in another recent study in *D. melanogaster* (21).

Evolution of Cleavage Drive-Resistant *tra* Alleles. While we found our system to be highly efficient for sex conversion in *D. melanogaster*, we noticed during routine screening of the stocks the appearance of female flies with the DsRed eye marker phenotype. This was contrary to our previous observation and expectations that all heterozygous (tra^{nCHE}/+) XX flies should develop at least an intersex phenotype. Two scenarios could explain the presence of females with the DsRed eye marker: (*i*) an aborted or imperfect HDR, during which the DsRed eye marker is copied faithfully while an essential part of the drive element was lost or mutated, which would result in a dead CHE allele (tra^{nD}), or (*ii*) the presence of an in-frame mutation in the *tra* allele, which abolishes the recognition site of the gRNA without affecting the function of the *tra*-encoded protein (tra^{Rst}). Such mutations are likely to emerge from in-frame indel mutations as a result of NHEJ events induced by the CHE itself.

To check these hypotheses, virgin females with the DsRed eye marker were isolated and individually crossed with w^- males. One of these crosses did not show any signs of an active drive system, with about 50% of the offspring showing the DsRed eye marker. Molecular analysis of the mother and some female

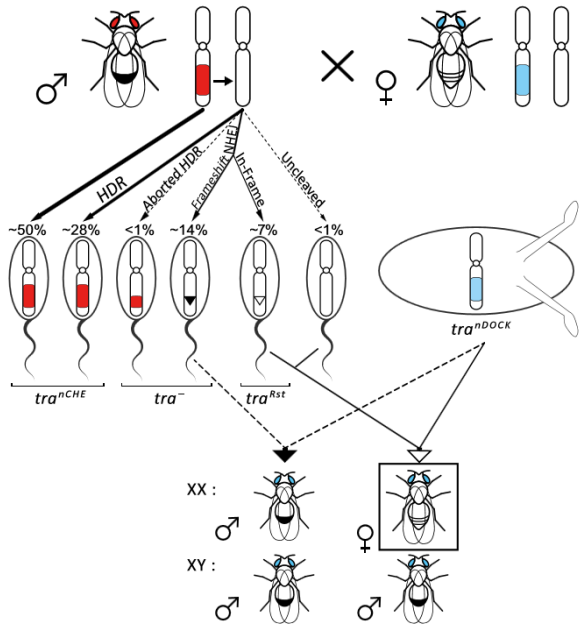

Fig. 2. CHE targeting of the homologous gene locus. By analyzing the ECFP/non-DsRed progeny of tra^{nCHE}/+ males and tra^{nDOCK}/+ virgins, we focused on non-HDR targeting events at a single *tra* locus. Estimation of each genotype frequency based on the observed efficiency values is indicated above sperm illustrations. Molecular analysis of the CHE target site in F_1 female progeny (boxed) identified independent NHEJ-derived in-frame indels that resulted in drive-resistant functional alleles (*SI Appendix*, Fig. S5*A*).

offspring from this cross revealed a large deletion in the CHE as the result of an aborted HDR event (*SI Appendix*, Fig. S4*A*). The other crosses, however, showed an efficient super-Mendelian inheritance, indicating the presence of an active CHE in the mother, which could be a sign of the presence of a tra^{Rst} allele in the mother. Sequencing the *tra* locus of these mothers confirmed the presence of in-frame indel mutations in the recognition site of the gRNA (similar to sequences in *SI Appendix*, Fig. S4*B*). By crossing such females carrying an active CHE with tra^{nCHE} males, we were able to obtain homozygous tra^{nCHE}/tra^{nCHE} males. When these homozygous males were crossed with w^- virgins, all offspring were either male or intersex (*SI Appendix*, Fig. S3*A*), which further confirmed the high sex-conversion efficiency of this *tra*-targeting CHE.

To further evaluate the drive-resistant allele hypothesis and to estimate the rate at which resistant alleles may emerge from NHEJ events, we crossed heterozygous virgins carrying the docking-null allele (tra^{nDOCK}/+) with heterozygous (tra^{nCHE}/+) driver males (Fig. 2). By looking at the progeny that carry the tra^{nDOCK} allele (marked by ECFP fluorescence) but lack a tra^{nCHE} allele (DsRed fluorescence), we confined our analysis to situations of non-HDR at the paternal wild-type *tra* allele. Sequencing the *tra* allele in non-DsRed, ECFP females of the first generation resulted in the discovery of various independent in-frame indel mutations (*SI Appendix*, Fig. S5*A*). This suggests that drive-resistant alleles, tra^{Rst}, are readily created as a result of NHEJ in heterozygous males that carry the CHE allele. To determine the frequency at which these tra^{Rst} alleles are generated, we crossed four heterozygous (tra^{nCHE}/+) males individually to tra^{nDOCK}/+ virgins and sequenced all progeny that showed only an ECFP fluorescence. We identified in-frame indels (tra^{Rst}) in up to 10% of all progeny, representing about one-third of all NHEJ events. The relative high emergence rate of such tra^{Rst} alleles in the F_1 progeny demonstrates the rapid evolution of resistance as a direct consequence of an active homing CHE (*SI Appendix*, Fig. S5*B*) and confirms similar results from other groups (21, 30).

Resistant Allele Dynamics and Spread. To estimate the dynamics of resistance allele emergence and spread in a population, we crossed (in five replicates each) w^- virgins with four different ratios of heterozygous tra^{nCHE}/+ males to w^- males and followed the progeny for up to 15 generations. Thereby we documented the sex ratios as well as the spread of the DsRed-marked tra^{nCHE} allele, whose presence in females indicates potential drive-resistant tra^{Rst} alleles (Fig. 3 and *SI Appendix*, Figs. S6–S8). The ratio of such DsRed-positive females increased progressively over the generations, corresponding to the expected selective increase of resistance allele frequency. To characterize the molecular basis of the resistance to HCGD, we sequenced the *tra* locus from DsRed-fluorescent females from all the experimental settings at generation F_6 and observed a diverse set of in-frame mutations representing drive-resistant tra^{Rst} alleles (*SI Appendix*, Fig. S4*B*). We also selected one setting for a molecular time-course analysis (setting D, replicate 4, at generations F_1, F_2, F_6, and F_{13}) and found that such mutations were heritable (*SI Appendix*, Fig. S5*C*). The diversity of these in-frame indels across experimental settings and generations shows that these tra^{Rst} alleles are constantly created, independently of each other, at the site of cleavage. Interestingly, we already had observed DsRed-fluorescent females at the F1 generation of this replicate. These possessed a wild-type *tra* allele (*SI Appendix*, Fig. S5*C*) but contained a large deletion in the *Cas9* gene of the tra^{nCHE} allele, which was likely the result of a rare, aborted HDR event (similar to that in *SI Appendix*, Fig. S4*A*). Following the populations to

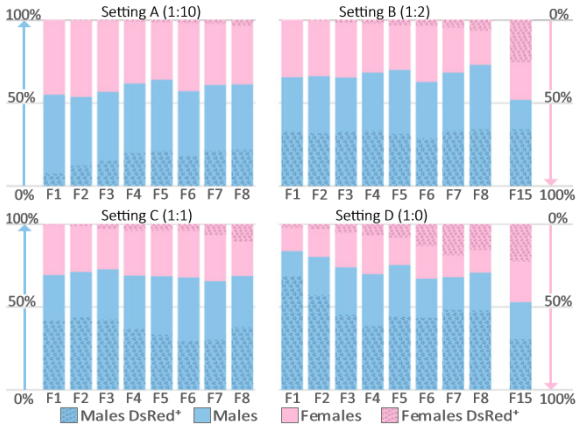

Fig. 3. Dynamics of sex ratio and indicated resistance allele spread in population experiments. w^- virgins were crossed with various ratios of CHE (tra^{nCHE}/+) and wild-type (w^-) males (settings A–D). For each setting five replicates were carried out (*SI Appendix*, Fig. S6). Progeny were screened for sex and the presence of the DsRed eye marker for up to 15 generations. In setting D, where only tra^{nCHE} males were used, a sex ratio of over 80% males was achieved within one generation, indicating the collapse potential of this forced male offspring-only system. In XX embryos, tra^{nCHE} attacks the wild-type *tra* locus in somatic cells, resulting in intersex individuals (*SI Appendix*, Fig. S2). Thus, only females carrying nonfunctional defective tra^{nCHE} or drive-resistant functional tra^{Rst} alleles can show the DsRed marker. Therefore, the DsRed marker serves as an indicator for the presence of the tra^{Rst} allele in females, and the rise in the percentage of DsRed females indicates the spread of resistance in the population. Screening the F_{15} progeny in settings B and D showed that the populations adapted to the presence of the tra^{nCHE} homing allele with the female sex ratio returning to about 50% (*SI Appendix*, Fig. S6).

generation F_{15}, we found an almost regular 1:1 ratio of males to females in all the replicates (Fig. 3 and *SI Appendix*, Fig. S8), independent of the original frequency of tra^{nCHE} allele inoculation (settings B and D).

A *tra*-Based Suppression Gene-Drive System for *C. capitata*. Having shown the capability of our proposed CHE in inducing sex conversion in *D. melanogaster* and after identifying potential weaknesses of the system due to resistance evolution, we simulated the outcome of using our proposed method as a pest-control strategy in *C. capitata* (Fig. 4). Our population dynamics simulation results indicate that the evolution of in-frame drive-resistant alleles at rates that we observed in *D. melanogaster* would indeed impede a population collapse in *C. capitata* (Fig. 4*A*), even if multiple releases were implemented in quick succession (Fig. 4*C*). To tackle the issue of in-frame drive-resistant alleles (these are problematic because they prevent homing while still allowing *tra* expression and hence are not removed due to a selective advantage), we considered the use of multiple gRNAs to target the *tra* gene to reduce the proportion of resistant alleles that are in-frame. Using multiple gRNAs may not have a drastic effect on the overall NHEJ rate but will reduce the in-frame resistant allele formation rate exponentially with each additional gRNA, as each new target site would have to obtain an in-frame mutation that does not affect the function of the protein (22, 31). Our simulation study predicts that by using multiple gRNAs, and thereby reducing the generation rate of in-frame resistance alleles by at least two orders of magnitude, the effectiveness of the system is greatly improved. A single release is still not sufficient to achieve a population collapse in *C. capitata* (Fig. 4*B*); however, three releases in quick succession are sufficient (Fig. 4*D*).

The above-mentioned simulations are for the scenario in which tra^{nCHE}/+; XX individuals are infertile intersexes; however, we also explored the case in which these individuals are fertile males (Fig. 4 *E* and *F*). In *D. melanogaster*, we observed that heterozygous (tra^{nCHE}/+) XX individuals develop into mosaic intersex individuals (*SI Appendix*, Fig. S3*A*). This is likely because the ectopic expression of *Cas9* under the control of the *Rcd-1r* promoter in only a proportion of the cells results in a mosaic phenotype. Since the intersex-based infertility of tra^{nCHE} XX individuals places a fitness load on the system and reduces the drive (by preventing its occurrence in XX individuals), we propose the use of an early embryonic promoter, such as *Sry-α*, for the expression of *Cas9*. Expression from *Sry-α* in germ cells will allow gene drive to occur, and the early blastoderm expression guarantees a uniform destruction of the wild-type *tra* allele in all cells of the embryo at a very early stage; therefore heterozygous (tra^{nCHE}/+) XX individuals could develop into fertile medfly males. This in turn reduces the fitness load associated with the drive allele and increases the drive (by allowing super-Mendelian inheritance of the drive allele to occur in tra^{nCHE}/+; XX individuals). Our simulation shows that enabling the fertility of heterozygous (tra^{nCHE}/+) XX individuals does enhance the effectiveness of the system in collapsing a *C. capitata* population following a single release, provided that the in-frame drive-resistant allele generation rate is reduced by using multiple gRNAs (Fig. 4 *E* and *F*). The tolerable generation rate of in-frame resistant alleles depends on the size of the targeted medfly population. Our simulations predict the extent by which this rate must be reduced to achieve a population collapse as a function of population size (*SI Appendix*, Fig. S9).

Discussion

Our mathematical modeling has shown that two main factors—the formation rate of the in-frame resistance allele and the fitness of heterozygous (tra^{nCHE}/+) sex-converted XX individuals—can have a significant effect on the expected outcome of a release in the wild. When heterozygous XX individuals are infertile, our model predicts that a population collapse can be achieved only if multiple

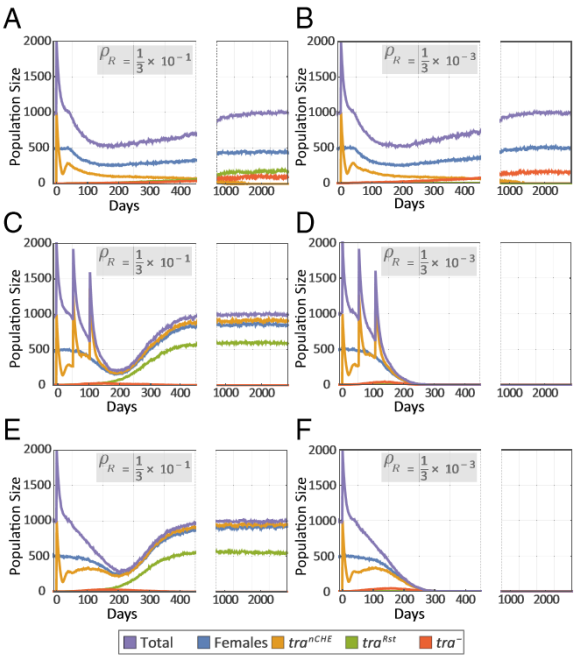

Fig. 4. Predicted dynamics of a Cas9-based homing system targeting the *tra* locus in *C. capitata*. Predictions are based on the population genetics model depicted in *SI Appendix*, Fig. S10 combined with the population dynamics model depicted in *SI Appendix*, Fig. S11 in which the life cycle of *C. capitata* is divided into four stages, egg, larva, pupa, and adult, with density-dependent mortality occurring at the larval stage (*SI Appendix*, Table S1). Homing occurs only in tra^{nCHE}/+ heterozygotes, where "+" represents the wild-type allele and "tra^{nCHE}" represents the intact drive allele. We assume a Cas9-mediated cleavage efficiency of 100% and a probability of accurate HDR following cleavage of 90% [NHEJ rate (δ) = 0.1]. By default, in-frame drive-resistant alleles (tra^{Rst}) account for one-third of generated resistant alleles, although this proportion may be reduced through gRNA multiplexing. The remaining cleavage-resistant alleles are out-of-frame or other mutations that result in a tra^{-}-null allele. The equilibrium population size of *C. capitata* is 1,000. Releases consist of 1,000 tra^{nCHE}; XY males at a single time or at intervals. In *A*–*D*, the scenario in which tra^{nCHE}; XX individuals are infertile intersexes is considered. (*A*) For a homing efficiency of 90% and an in-frame resistant allele generation rate ($\rho_R = \delta\theta$, where δ is the NHEJ rate and θ is the fraction of NHEJs that produce in-frame indels) of one-third of 10%, a single release of 1,000 tra^{nCHE}; XY males results in temporary population suppression, halving the adult population size, with the population rebounding over a period of several years. (*B*) Decreasing the in-frame drive-resistant allele generation rate, ρ_R, by two orders of magnitude to 1/300 of 10%, and hence increasing the out-of-frame resistant allele generation rate, $\rho_B = \delta(1-\theta)$, to ∼10%, the population suppression is still only moderate and transient. (*C*) If three releases of 1,000 tra^{nCHE}; XY males are carried out in succession, the extent of population suppression is much greater (>75% suppression); however at a ρ_R of one-third of 10%, the population still rebounds over a period of several years with an increase in the frequency of tra^{Rst} alleles. (*D*) If three consecutive releases are carried out for a construct with the decreased in-frame drive-resistant allele generation rate, population elimination can be achieved within ∼1 year after the last release. (*E* and *F*) The scenario in which tra^{nCHE}; XX individuals are fertile males is considered. For a homing efficiency of 90% and an in-frame resistant-allele generation rate, ρ_R, of one-third of 10%, a single release of 1,000 tra^{nCHE}; XY males results in temporary population suppression, as in-frame drive-resistant alleles become prevalent, preventing population elimination (*E*). However, if the in-frame resistant-allele generation rate, ρ_R, is reduced by two orders of magnitude, to 1/300 of 10%, the emergence of in-frame drive-resistant alleles is unlikely, and the population can be eliminated following a single release of 1,000 tra^{nCHE}; XY males (*F*).

inundative releases of the driver males are performed (Fig. 4*D*). While this limitation could potentially be overcome by using an early embryonic stage promoter such as *Sry-α* (Fig. 4*F*), this may not be desirable to ensure the local treatment of an insect pest population without the potential concern about the elimination of an entire species. Similar considerations have also been brought forward by Prowse et al. (32) with respect to fighting invasive vertebrate species.

In addition, we have shown that in our system it is the generation rate of in-frame drive-resistant alleles, rather than the overall NHEJ rate, that has a significant impact on the outcome of release scenarios. This is of significant importance for species such as *D. melanogaster* (and possibly for related pest species such as *Drosophila suzuki*) that might seem semirefractory toward homing-based gene-drive strategies (27), as it indicates that future designs may not necessarily require an extremely high homing rate but that only lowering the formation rate of the in-frame resistance allele and thus employing multiple gRNAs (22, 31) might be sufficient for an efficient suppression gene-drive strategy in such species.

Our results support the idea that using a CHE to target genes that are essential for female-specific development in insects, such as *tra*, can effectively result in a gender-biased population, finally resulting in a population collapse. This provides a basis for further development of similar suppression gene-drive strategies to introduce a gender bias in wild populations of insect pests such as the medfly or disease vectors. If such a gender bias can be sustained long enough, species-specific elimination of the target species can be achieved. If the HCGDs explored here were applied to efficient pest-control management, the strategy in which tra^{nCHE}/+; XX individuals are infertile intersexes is safer, because it requires multiple releases to achieve population collapse and hence will cause a population collapse only where these releases are carried out.

Overall, we provide here an example, an implementation strategy, and the mathematical modeling required for the design and optimization of a homing-based sex-conversion suppression gene-drive approach for local or global species-specific elimination of insect pest or disease vector species. Moreover, we show that only lowering the formation rate of in-frame drive-resistant alleles by employing multiple gRNAs may be sufficient to achieve an effective suppression gene-drive outcome, which has important implications for the design of such systems in species that exhibit a low homing rate in their germ cells.

Materials and Methods

Detailed methods on cloning, transgenesis, screening, molecular analysis, stock keeping of *D. melanogaster* strains, population modeling, and simulations can be found in *SI Appendix, SI Materials and Methods*.

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Supplementarty Information

Consequences of resistance evolution in a Cas9-based sex conversion suppression gene drive for insect pest management

Short title: Effects of resistance on sex conversion gene drive

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SI Materials and Methods

Computational modeling of gene drive in *D. melanogaster*. Equation B4 from the model by Unkless et. al. for resistance evolution in gene drive (1) was expanded as below to include all the important parameters of our study, where x_w, x_d, x_r and x_b are allele frequencies for wild-type, driver, in-frame resistance, and frameshift alleles, superscripts represent the sex. Parameters *c*, δ, and ϑ represent cleavage rate, NHEJ rate, and fraction of NHEJs that produce in-frame indels, respectively. ω_{ab} shows the fitness of an individual with genotype ab.

$$\overline{\omega}(t) = \left(x_w^m x_d^f + x_d^m x_w^f\right)[(1-c)\omega_{dw} + c(1-\delta)\omega_{dd} + c\delta(1-\theta)\omega_{db} + c\delta\theta\omega_{dr}] + \left(x_w^m x_r^f + x_r^m x_w^f\right)\omega_{rw} + \left(x_w^m x_b^f + x_b^m x_w^f\right)\omega_{bw} + \left(x_d^m x_r^f + x_r^m x_d^f\right)\omega_{dr} + \left(x_d^m x_b^f + x_b^m x_d^f\right)\omega_{db} + \left(x_b^m x_r^f + x_r^m x_b^f\right)\omega_{rb} + \sum_i x_i^m x_i^f \omega_{ii} \quad \text{(E1)}$$

For the specific case of *D. melanogaster* where ω_{dw}, ω_{dd}, ω_{db}, and ω_{bb} are 0 for females and all other fitness values are considered 1, the following equations can be derived from equation E1 to recursively calculate the allele frequencies at each generation for females:

$$x'^f_d = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_d^m x_r^f + x_r^m x_d^f\right)}{2\bar{\omega}_{Dm}(t)} \quad \text{(E2)}$$

$$x'^f_r = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right) + 2x_r^m x_r^f}{2\bar{\omega}_{Dm}(t)} \quad \text{(E3)}$$

$$x'^f_b = \frac{\left(x_w^m x_b^f + x_b^m x_w^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right)}{2\bar{\omega}_{Dm}(t)} \quad \text{(E4)}$$

$$x'^f_w = \frac{\left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_w^m x_b^f + x_b^m x_w^f\right) + 2x_w^m x_w^f}{2\bar{\omega}_{Dm}(t)} \quad \text{(E5)}$$

and for males:

$$x'^m_d = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)[c(1-\delta)+1] + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_d^m x_b^f + x_b^m x_d^f\right) + 2x_d^m x_d^f}{2\bar{\omega}_{Dm}(t)} \quad \text{(E6)}$$

$$x'^m_r = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right) + 2x_r^m x_r^f}{2\bar{\omega}_{Dm}(t)} \quad \text{(E7)}$$

$$x'^{m}_{b} = \frac{\left(x^{m}_{w}x^{f}_{d}+x^{m}_{d}x^{f}_{w}\right)[c\delta(1-\theta)]+\left(x^{m}_{w}x^{f}_{b}+x^{m}_{b}x^{f}_{w}\right)+\left(x^{m}_{d}x^{f}_{b}+x^{m}_{b}x^{f}_{d}\right)+\left(x^{m}_{b}x^{f}_{r}+x^{m}_{r}x^{f}_{b}\right)+2x^{m}_{b}x^{f}_{b}}{2\bar{\omega}_{Dm}(t)}$$

(E8)

$$x'^{m}_{w} = \frac{\left(x^{m}_{w}x^{f}_{d}+x^{m}_{d}x^{f}_{w}\right)(1-c)+\left(x^{m}_{w}x^{f}_{r}+x^{m}_{r}x^{f}_{w}\right)+\left(x^{m}_{w}x^{f}_{b}+x^{m}_{b}x^{f}_{w}\right)+2x^{m}_{w}x^{f}_{w}}{2\bar{\omega}_{Dm}(t)}$$

(E9)

where

$$\bar{\omega}_{Dm}(t) = \left(x^{m}_{w}x^{f}_{d}+x^{m}_{d}x^{f}_{w}\right)(1+c\delta\theta)+2\left(x^{m}_{w}x^{f}_{r}+x^{m}_{r}x^{f}_{w}+x^{m}_{w}x^{f}_{b}+x^{m}_{b}x^{f}_{w}+x^{m}_{d}x^{f}_{r}+x^{m}_{r}x^{f}_{d}+x^{m}_{b}x^{f}_{r}+x^{m}_{r}x^{f}_{b}+x^{m}_{w}x^{f}_{w}+x^{m}_{r}x^{f}_{r}\right)+x^{m}_{d}x^{f}_{b}+x^{m}_{b}x^{f}_{d}+x^{m}_{d}x^{f}_{d}+x^{m}_{b}x^{f}_{b}$$

(E10)

Guide RNA selection and HRMA. Several gRNAs targeting the first exon of the *D. melanogaster tra* locus were selected using CRISPR DESIGN online tool. Selected guide sequences (*SI Appendix*, Fig. S1*B*) were cloned into the pCFD2 plasmid (2) (Addgene 49409, gift from S. Bullock) using annealed oligonucleotides for each gRNA (*SI Appendix*, Fig. S1*A*). One hour old embryos from the act5-cas9 *D. melanogaster* strain (2) (Bloomington 54590) were injected using the construct for each gRNA. After 24 hours, individual embryos were homogenized in 50 µl of smashing buffer (10 mM Tris-HCl, pH 8.2, 25 mM NaCl, 1mM EDTA, 0.2% Triton x100 and 200 µg/ml Proteinase K) (3) using small tips. Samples were kept at 55 °C for 1 hour followed by 5 minutes incubation at 95°C to heat inactivate Proteinase K. 1 µl was used for PCR using MK078 and MK079 primers (*SI Appendix*, Table S2) with EvaGreen qPCR master mix and High Resolution Melt curves (4) were obtained in a BioRad CFX96 real-time C1000 thermal cycler at 0.2°C steps. Relative fluorescent at 75°C and 85°C were normalized to 1 and 0, respectively, and the control graph was subtracted from all the guide RNA graphs. The guide that showed the largest difference (g4) was selected as the most efficient guide RNA. To make sure that off-target effects are minimal (4), three of the top similar sequence hits from the CRISPR DESIGN online tool (5) were checked for the most efficient guide (g4) using High Resolution Melting Analysis (HRMA) employing MK155/MK156, MK157/MK158 and MK159/MK160 primer pairs (*SI Appendix*, Table S2).

Constructs. To generate the SG022 plasmid (pCRII-tra4R-attP-3xP3CFP-attP-tra4L, GeneBank KY171964), In-Fusion assembly (Clontech, USA) was performed on HindIII/ApaI digested pCRII (ThermoFisher, USA) backbone and PCR products of (i) MK024/MK122 primers on genomic DNA from the *D. melanogaster* strain Oregon-R for left homologous arm, (ii) MK117/MK116 primers on pBac{3xP3-ECFPaf} (6) for 3xP3-ECFP-SV40pA, and (iii) MK123/MK025 primers on genomic DNA from the Oregon-R strain for right homologous arm. MK116 and MK117 primers (*SI Appendix*, Table S2) introduce *attP* sites (7) at both ends of the ECFP marker to generate a recombinase-mediated exchange cassette. The SG012 plasmid (pCFD3-g4) was generated by cloning annealed MK083 and MK044 oligonucleotides into the pCFD3 plasmid (2) (Addgene 49410, gift from S. Bullock) using the depositor's suggested protocol (8). To generate the SG011 plasmid (pCRII-attBSmaI), the pCRII vector was first digested with XbaI/HindIII followed by ligation of annealed MK060 and MK061 oligonucleotides (*SI Appendix*, Table S2). Then the NsiI cut site in the vector was destroyed by first digesting the plasmid with NsiI followed by T4 DNA polymerase treatment and religation using T4 DNA Ligase. In-Fusion assembly was performed on the BamHI/NotI digested pIE4 plasmid (9) and PCR products of (i) MK072/MK075 primers (*SI Appendix*, Table S2) on genomic DNA from Oregon-R strain for first intron of *alpha-tub84B* and (ii) MK076/MK077 primers on pBS-Hsp70-Cas9 (Addgene 46294, gift from M. Harrison, K. O'Connor-Giles, J. Wildonger) for *D. melanogaster* codon optimized SpCas9 coding sequence, to generate the SG020 plasmid (pIE4-aTubIGT1-Cas9). MK134 and MK135 primers (*SI Appendix*, Table S2) were used to amplify the aTubI1-Cas9 fragment from SG020 plasmid. The fragment was directly ligated to SmaI digested SG011 to generate the SG023 plasmid (pCRII-attB-aTubCas9-

attB). For the SG024 plasmid (pCRII-attB-aTubCas9bTub-U63g4-DsRed-attB, GeneBank KY171962), in-Fusion assembly was performed on AscI digested SG023 plasmid and PCR products of (i) MK144/MK145 primers on genomic DNA from Oregon-R strain for the 3' UTR of beta-tub85D (10), (ii) MK147/MK146 primers on SG012 for U6:3-g4, and (iii) MK149/MK148 primers on pBac{3xP3-DsRedaf} (11) for 3xP3-DsRed-SV40pA. To amplify each of the promoters Oregon-R genomic DNA was used as template and (i) MK140/MK141 primer pairs (*SI Appendix*, Table S2) were used to amplify a 900 bp fragment of the Rcd-1r promoter (10), (ii) MK142/MK143 primer pairs (*SI Appendix*, Table S2) were used to amplify a 550 bp fragment of the *Sry-α* promoter (12), and (iii) MK138/MK139 primer pairs (*SI Appendix*, Table S2) were used to amplify a 500 bp fragment of the *DNApol-α180* promoter (13). The PCR products were then digested using XbaI (or AvrII) and XhoI restriction enzymes and the products were ligated to AvrII/XhoI digested SG024 plasmid to generate SG039 (pCRII-attB-Rcd1rp-aTubCas9bTub-U63g4-DsRed-attB, GeneBank KY171963), SG040 (pCRII-attB-sryap-aTubCas9bTub-U63g4-DsRed-attB), and SG037 (pCRII-attB-DPol180p-aTubCas9bTub-U63g4-DsRed-attB) plasmids respectively. MK153 and MK154 primers (*SI Appendix*, Table S2) were used to amplify the phiC31 coding sequence from the plasmid pcDNA3.1-phiC31 (14) (Addgene 68310, gift from K. Basler). The PCR product was then digested with BsaI and NotI restriction enzymes and was ligated to NcoI/NotI digested pSL[faHSfa] plasmid (15) to generate the SG042 helper plasmid (pSL-DmHsp70-phiC31-Hsp70).

Generation of the docking (*tra^{nDOCK}*) strain. To generate the docking strain (*SI Appendix*, Fig. S2*A*), *tra^{nDOCK}*, 30 minutes old de-chorionated embryos from the act5-Cas9 *D. melanogaster* strain (2) were covered with hydrocarbon oil (Voltalef 10S) and injected with an injection mix containing 500 ng/µl of SG022 (pCRII-tra4R-attP-3xP3CFP-attP-tra4L) HDR donor plasmid and 300 ng/µl of SG012 (pCFD3-g4) gRNA-producing plasmid. Embryos were kept humid at 25°C for 24 hours on an apple agar plate. Newly hatched larvae were gently collected from the apple agar plate using a size 00 brush and placed on *D. melanogaster* food supplemented with dried yeast in a small vial. Larvae were kept at 25°C until eclosion and only male offspring were individually crossed with *w*$^{-}$ virgins. F_1 third instar larvae from individual vials were collected from the food by applying CO_2 to the media. The larvae were aligned on a cold microscope slide and screened under a Zeiss fluorescent binocular for the ECFP eye marker. Positive larvae from each cross were placed into new vials until eclosion. Individual male flies with the ECFP eye marker were then crossed with virgins from the $\left[\frac{w^-}{w^-}; \frac{CyO\ (Cy)}{Sp}; \frac{TM3(Sb)}{Dr}\right]$ balancer strain. F_2 flies carrying the ECFP eye marker, *Cy* (*Curly wings*) and *Sb* (*Stubble*) phenotypes were self-crossed. F_3 flies carrying the ECFP eye marker and the *Sb* phenotype without any of the other balancer phenotypes were kept to establish the strain. Molecular analysis was performed to verify the proper genome editing at the third chromosomal *tra* locus that generated *tra^{nDOCK}*.

Molecular Characterization of *tra^{nDOCK}* strain. To confirm the correct integration of the docking cassette into the *tra* locus, MK126/MK128 and MK127/MK129 primer pairs (*SI Appendix*, Table S2) were used to amplify products of ~3 kbp from the *tra^{nDOCK}* genomic DNA. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (3) using small tips. MK126 and MK127 primer binds to regions upstream and downstream of the Left and Right Homologous Arms used for the HDR, respectively. MK128 and MK129 primers, however, bind to 3' end and 5' end of the ECFP CDS, respectively. Therefore, a product of around 3 kbp from each of these primer pairs can only form if integration at the correct locus has occurred. Off-target integration does not result into an amplification product with these primer pairs because MK126 and MK127 primers bind outside the homologous arms regions. One of the fly strains that passed all of the quality control criteria was then kept as a stock.

Generation of the homing *tra^{nCHE}* strain. To generate the homing *tra^{nCHE}* strains (*SI Appendix*, Fig. S2*B*), 30 minutes old embryos de-chorionated from the docking strain (*tra^{nDOCK}*) were covered with hydrocarbon oil and injected with an injection mix containing 500 ng/µl of each of the donor plasmids for respective promoters (SG039, SG040, and SG037) and 300 ng/µl of SG042 (pSL-DmHsp70-phiC31-Hsp70) helper plasmid to perform a Recombinase-Mediated Cassette Exchange (RMCE)(16). Embryos were kept humid at 25 °C for 24 hours on an apple agar plate. Newly hatched larvae were gently collected from the apple agar plate using a size 00 brush and placed on *D. melanogaster* food supplemented with dried yeast in a small vial. Larvae were kept at 25°C until

eclosion. Newly eclosed flies were individually crossed with w^- flies. For each of the promoters F_1 offspring was screened for the absence of ECFP and the presence of DsRed eye marker. Individual males with only the DsRed eye marker were then crossed with w^- virgins. Positive F_2 individuals (consisting of males/intersexes only) from each single cross were then pooled together and w^- virgins were added to the pool. Few of the F_2 males/intersexes were used for DNA extraction for molecular characterization and verification of RMCE. To sustain the strain over generations, a few w^- virgins were added to the vials every two weeks.

Molecular Characterization of Rcd-1r *tra^{nCHE}* strains. To confirm the fidelity of the RMCE and also to determine the orientation of the integration, MK073/MK078 and MK073/MK079 primer pairs (*SI Appendix*, Table S2) were used to amplify a ~2 kbp region from the *tra^{nCHE}* genomic DNA. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (3) using small tips. MK073 binds to the upstream of the SpCas9 coding sequence within the CHE. MK078 and MK079 bind to upstream and downstream of the g4 target site, respectively. A 2 kbp amplification product with MK073/MK078 primer pairs indicates a sense integration event (endogenous *tra* and SpCas9 CDSs on the same strand). However, a 2 kbp amplification product with MK073/MK079 primer pairs indicates an anti-sense integration event (endogenous *tra* and SpCas9 CDSs on opposite strands). One of the fly strains with an anti-sense integration that passed all the quality control criteria was kept as a stock and was used for all further experiments.

Stock keeping of *tra^{nCHE}* strains. Despite the fact that the high cost of drive in our *tra^{nCHE} D. melanogaster* strains does not allow for spread of the drive allele in any population and thus serves as a very effective biological confinement strategy (Fig. 1 C and D), we carried out stock keeping of this homing *tra^{nCHE}* strain as well as all experiments generating and using this strain with utmost care to not have potential gene drive individuals escape the laboratory. All experiments were performed in our well-equipped safety level one (S1) laboratory, but only in windowless internal rooms, with ventilation in- and outlets covered with tight mesh and doors supplied with brushes. Flies were always anesthetized before opening containers as well as analyzed and sorted under constant anesthetization. Fly traps were installed in the rooms and in the neighboring corridors. For transport between rooms, flies were put in double-walled containers. Before discarding, flies were finally deep frozen for more than twelve hours. Despite the design and commencement of our study in 2013, it already met the criteria for safeguarding gene drive experiments in the laboratory as published in Science in 2015 (17) since two stringent confinement strategies were employed: (i) biological confinement based on the high cost of this specific gene drive in *D. melanogaster* and (ii) organizational as well as physical containment based on the rules for handling this strain in our S1 laboratory. In 2016, we were informed of the new guidelines on handling gene drive experiments in Germany by the German Central Commission for Biological Safety (ZKBS) that suggested to perform such experiments only in safety level two (S2) laboratories. Therefore, we stopped our experiments and moved our *tra^{nCHE}* strain temporarily to a neighboring S2 laboratory for stock maintenance only. At the same time we applied for a single case evaluation of our experiments with the ZKBS that approved of further handling the *tra^{nCHE}* strain in our S1 laboratory with the above mentioned measures (file reference: ZKBS 45110.1933). After publication of this study, we will discard the strain that can easily be re-generated by RMCE from the docking line *tra^{nDOCK}*.

Estimating the sex conversion and homing efficiencies. The sex conversion efficiency of the *tra^{nCHE}* allele was evaluated by crossing twelve homozygous *tra^{nCHE}/tra^{nCHE}* males individually with three w^- virgins each. The offspring was then screened for sex and kept to inbreed to check for any sign of fertility of potential not-recognized F_1 females. All inspected F_1 progeny was male or intersex and no F_2 progeny was observed. To estimate the homing efficiency of the *tra^{nCHE}*, 12 heterozygous *tra^{nCHE}/+* males were individually crossed with three w^- virgins each. The offspring was then screened for sex and the presence of DsRed fluorescent eye marker under a Zeiss fluorescent binocular (*SI Appendix*, Fig. S3 *B* and *C*). The homing efficiency was calculated as the ratio of targetable alleles that had been successfully homed by the *tra^{nCHE}* allele, by equation E11, assuming an equal segregation of chromosomes in male gametes.

$$\frac{DsRed^{+}-(Total/2)}{Total/2} \qquad \text{(E11)}$$

Estimating the targeting efficiency. To estimate the targeting efficiency of our CHE, heterozygous *tra*^{nCHE}/+ males were crossed with heterozygous *tra*^{nDOCK}/+ virgins (Fig. 2) – both in batch and in single crosses with individual *tra*^{nCHE} males. The F_1 offspring was then screened for eye marker and only individuals carrying the ECFP eye marker but not the DsRed eye marker were selected for molecular analysis. MK058/MK059 primer pairs (*SI Appendix*, Table S2) were used to amplify a ~750 bp region which was used for Sanger sequencing using the MK058 primer. Sequencing results were analyzed using Geneious software (Biomatters, New Zealand). Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (3) using small tips.

Population experiments. Population experiments were performed in four settings A–D with five replicates for each of the settings (*SI Appendix*, Fig. S6*A*). To avoid any bias in mating, we made sure all flies were of the same age and before putting them together in the same vial all flies were kept anesthetized using CO_2. After 72 hours of mating time, a 3–6 hours egg lay collection was obtained, after which all parents were removed and were kept frozen at -80 °C. For each following generation, all progeny of the 3–6 hour egg collection was allowed to eclose and then used as parents to set up the next generation. Again after a 3–6 hours egg lay, these parents were removed and used for screening their sex and the presence of the DsRed eye marker prior to freezing and storage at -80 °C. The 3-6 hour egg collection was based on the amount of time that is necessary for the population of parents to lay enough eggs to generate a progeny size which fills but not over-populates a medium size fly food vial (Diameter: 36 mm; Height: 82 mm). At each generation, enough time was given to all the pupae to eclose followed by 72 hours of mating to allow for a representative population in the next generation. For each egg collection, all parents as well as all the respective progeny were screened.

Molecular analysis of *tra* loci and aborted HDR *tra*$^{-}$ alleles. MK058 and MK059 primers (*SI Appendix*, Table S2) were used to PCR-amplify a ~750 bp fragment surrounding the g4 target site. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (3) using small tips. Samples were kept at 55°C for 1 hour followed by 5 minutes incubation at 95°C to heat inactivate the Proteinase K. 5 µl of samples were used for PCR using Phusion DNA polymerase in 50 µl by 2 minutes incubation at 96°C followed by 40 cycles of 96°C for 20", 70°C for 30", and 72°C for 40" with 5 minutes final extension at 72°C. PCR products were then sent for Sanger sequencing using primer MK058. Sequencing results were analyzed using Geneious software (Biomatters, New Zealand). MK146 and MK134 primers (*SI Appendix*, Table S2) were used to PCR amplify a ~6 kbp fragment consisting of the U6:3-g4 and SpCas9 CDS from DsRed female's genomic DNA. PCR products were analyzed by agarose gel electrophoresis and fragment sizes were compared with the PCR product of same primer pairs on genomic DNA of a male individual from the stock as reference. The reduction in size of the fragments served as an indication of an aborted HDR (*SI Appendix*, Fig. S4*A*). To confirm the results at the molecular level, PCR products were then used for Sanger sequencing using MK085, MK086, MK087, and MK088 primers (*SI Appendix*, Table S2).

Population genetic model for homing system targeting the *tra* locus in *C. capitate*. To model the potential application of the homing-based gene drive system targeting the *tra* locus to suppress populations of the medfly, *C. capitata*, we combined a population genetic model describing the inheritance pattern of the homing system (*SI Appendix*, Fig. S10) with a population dynamic model described previously (18) adapted for the *C. capitata* (the original model describes the population dynamics of the main African malaria vector, *An. gambiae*) (*SI Appendix*, Fig. S11).

In the population genetic model (*SI Appendix*, Fig. S10), we describe the offspring genotype distribution in *C. capitata* for each combination of maternal and paternal genotypes for a homing-based gene drive system targeting the *tra* locus. We denote the wild-type *tra* allele by "W", the drive allele, *tra*^{nCHE}, by "D", a drive-resistant allele with an in-frame indel at the *tra* locus, *tra*^{Rst}, by "R", and a drive-resistant null allele, *tra*$^{-}$, by "B". Homing is only manifest in WD heterozygotes, whereby WD individuals produce D gametes in the germline at a frequency equal to $(1+e)/2$, where e denotes the "homing efficiency," which represents the proportion of W gametes that are converted into D gametes through the act of homing. Homing efficiency, e, is equal to the

product of the cleavage rate, c, and the probability of accurate homology-directed repair (HDR) given that cleavage has occurred, P_{HDR}, i.e. $e=cP_{HDR}$. Wild-type alleles may also be produced by WD individuals at a rate equal to $(1-c)/2$; however it should be noted that the rate at which this occurs has been shown to be negligible in a recent analysis (19) and hence this possibility is not depicted in the crosses in *SI Appendix*, Fig. S10. Homing-resistant alleles, which result from W alleles that are cleaved but for which accurate HDR does not occur, are produced at a frequency equal to $c\delta/2$, where $\delta=(1-P_{HDR})$. A proportion, θ, of these resistant alleles are in-frame indels (R), for which expression of functional *tra* gene is retained, and the remainder, $(1-\vartheta)$, are out-of-frame or other mutations (B), for which the function of *tra* gene is disrupted. The proportion of W gametes that are converted to R or B gametes is therefore given by $\rho_R=c\delta\vartheta$ and $\rho_B=c\delta(1-\vartheta)$, respectively. The proportion of generated resistant alleles that are in-frame indels is expected to be ~1/3, although this proportion can be reduced through multiplexing. All other individuals produce gametes at standard Mendelian frequencies. In this system, there are six possible fertile female genotypes – DR/XX, RR/XX, RB/XX, WR/XX, WB/XX and WW/XX – and 14 possible fertile male genotypes – DD/XY, WD/XY, DR/XY, DB/XY, RR/XY, RB/XY, BB/XY, WR/XY, WB/XY, WW/XY, DD/XX, WD/XX, DB/XX and BB/XX (*SI Appendix*, Fig. S10).

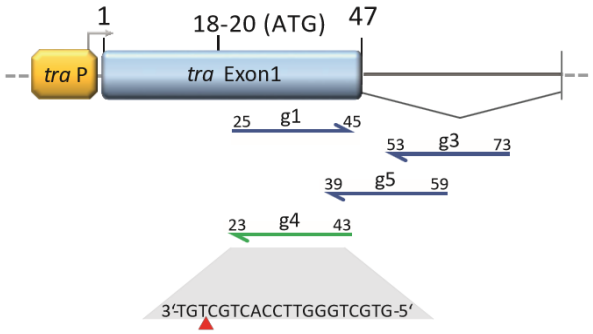
Population dynamic model for *C. capitate*. In the adapted population dynamics model (*SI Appendix*, Fig. S11), the medfly life cycle is divided into four life stages – egg, larva, pupa and adult (both male and female adults are modeled) (*SI Appendix*, Fig. S10). The durations of the juvenile stages differ (*SI Appendix*, Table S1) but their daily, density-independent mortality rates are assumed to be identical and are chosen for consistency with the population growth rate in the absence of density-dependent mortality. The intrinsic population growth rate of medfly populations in the absence of density-dependent mortality, r_M, was calculated from average monthly trap catch data across four sites in Western Cape Province, South Africa between the months of December 2009 and March 2010 and shown to be consistent with a population growth rate of $r_M=1.031$ per day (20). Additional density-dependent mortality occurs at the larval stage, and we used a density-dependent equation of the form, $F(L)=\sqrt[T_L]{\alpha(\alpha+L)}$, where L is the number of larvae, T_L is the duration of the larval stage, and α is a parameter influencing the strength of density-dependence. Adult males mate throughout their lifetime, while adult females mate only once, soon after that they mature. For these simulations, we assumed fecundity rates to be consistent between genotypes, with fertile females laying β eggs per day. Initial estimates for these and other parameter values are provided in *SI Appendix*, Table S1, and the equations describing the equivalent implementation of this model for *An. gambiae* are included in Supplementary File S1 of (18).

We used a stochastic implementation of this model to capture random effects at low population sizes, for instance when the CRISPR-based homing system is causing significant population suppression. We assume the number of eggs produced per day by females follows a Poisson distribution, the number of eggs having each genotype follows a multinomial distribution according to the parental genotypes and inheritance pattern, and all survival/death events follow a Bernoulli distribution. Finally, female mate choice follows a binomial distribution with probabilities given by the relative frequency of each male genotype in the population.

Experimental design and statistics. All individual data points are displayed in supplementary figures with mean and s.d., and sample size for all experiments are mentioned in the main text and figures as appropriate. No power calculations were performed to estimate the sample size. No randomization or blinding was performed, for each cross all progeny was screened. Replicate numbers for estimation of efficiencies are consistent with other similar gene drive studies in insects. A normal probability plot was generated for obtained homing efficiencies of each replicate to ensure the normal distribution of data points. In crosses with individual heterozygous tra^{nCHE} flies, replicates that did not result in progenies were excluded. These were most likely a result of a cross between an infertile intersex fly which are often indistinguishable from true males. In crosses between individual heterozygous tra^{nCHE} males with heterozygous tra^{nDOCK} virgins, two of the replicates that showed a 1:1 ratio of $DsRed^+$:$DsRed^-$ offspring were not used for molecular analysis and sequencing. All statistical analyses were performed on Microsoft Excel 2007. Plots were generated using GraphPad Prism 7 and R.

SI Figures

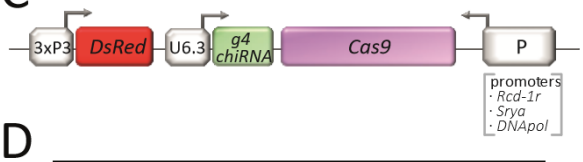
A



B

Plasmid	Guide ID	Seed Sequence	Oligo 1	Oligo 2
SG002	g1	GGCAGTGGAACCCAGCATCG	MK037	MK038
SG004	g3	GATCGGTTATACTATATAGT	MK041	MK042
SG005	g4	GTGCTGGGTTCCACTGCTGT	MK043	MK044
SG006	g5	GATAGTGGGTACCTCGATGC	MK048	MK049

C



D

Female Offspring [%]				
Replicate	tra^{nDOCK} Control	Promoter in tra^{nCHE} *Rcd-1r*	*Sry-α*	*DNApol*
1	56.14	20.76	20.33	3.96
2	54.60	23.11	15.85	20.49
3	49.11	12.76	8.99	4.27
4	51.18	10.79	22.22	17.68
5	53.51	2.34	10.40	7.77
6	52.78	15.27	24.39	14.83
7	49.30	11.22	0	8.61
8	42.22	14.06	0	15.00
9	45.08	0	14.62	10.24
10	39.07	17.18	N/A	30.09
Average	49.30	12.75	12.98	13.29

N/A: no offspring/ unsuccessful cross

Fig. S1. Generation of a homing CHE for the *D. melanogaster tra* locus.

(**A**) Four gRNAs targeting the first exon of the *D. melanogaster tra* locus with g4 (green) identified as the most efficient gRNA using HRMA (numbers indicate bases after transcription start site). (**B**) Oligos (*SI Appendix*, Table S2) used for the generation of gRNA plasmids. (**C**) Structure of the CHE used in this study. Human codon optimized *SpCas9* coding sequence is placed under the control of each of the three promoters under study. *Rcd-1r* germline specific promoter, *Sry-α* early zygotic promoter, and *DNApol-α180* cell cycle dependent promoter. g4 guide RNA is expressed by *U6:3* promoter. A 3xP3 driven DsRed eye marker is used to allow for screening and identification of strains. (**D**) To evaluate the drive as well as somatic sex conversion efficiency of each of the promoters, individual heterozygous tra^{nCHE} males from each of the three CHE variants (*Rcd-1r*-CHE, *Sry-α*-CHE, and *DNApol-α180*-CHE) were crossed with *w-* virgins and offspring were screened for their sex. tra^{nDOCK} strain was used as control. The very low frequency of female offspring clearly indicate that all three of these promoters are capable of inducing somatic sex conversion in *D. melanogaster*. Assuming an equal distribution of X and Y chromosomes and a sex conversion rate of 100% in XX embryos receiving a tra^{nCHE} allele from their father, a homing rate of ~49% can be calculated for each of the these promoters from the below equation which indicates all three of these promoters had equally well been able to successfully perform homing in the germline of the heterozygous tra^{nCHE} males and drive into the next generation.

Homing efficiency = $\frac{E_f^{'} - O_f}{E_f^{'}}$

Where O_f is the observed female frequency and $E_f^{'}$ is the expected female frequency in the absence of gene drive and 100% sex conversion of heterozygous XX individuals, which equates to 25% if an equal distribution of X and Y chromosomes is assumed.

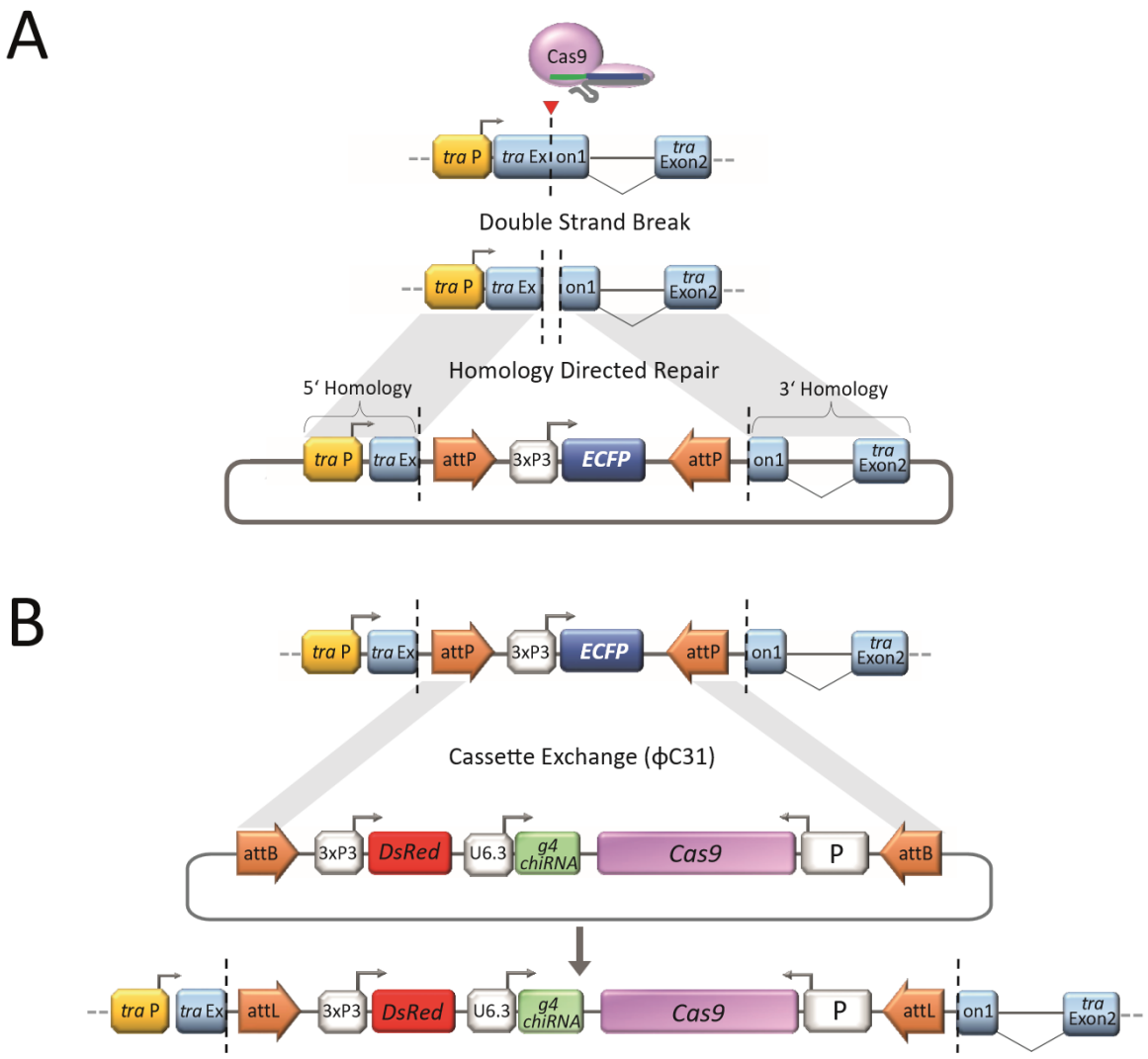


Fig. S2. Transgenesis strategy for integration of CHE in *D. melanogaster.*

(**A**) Using the g4 gRNA and Cas9, a ϕC31 RMCE docking cassette containing an eye-specifically driven (*3xP3*) *cyan fluorescent marker* (*ECFP*) CDS flanked by *attP* sites was integrated into the 1st exon of the *tra* locus to generate the docking null allele tra^{nDOCK}. (**B**) A cassette – containing the coding sequence of *SpCas9* endonuclease (*Cas9*, under the control of *Rcd-1r* germline specific promoter), the *U6.3* driven chimeric gRNA (*g4 chiRNA*), and a red *fluorescent eye marker* (DsRed) CDS – was used to replace the *ECFP* marker CDS by ϕC31 -mediated RMCE to generate the homing CHE null allele tra^{nCHE}.

A

male female mosaic intersex

B

Total No. Flies	Male		Female		Male Frequency	DsRed⁺ Frequency	Homing Efficiency
	DsRed⁺	DsRed⁻	DsRed⁺	DsRed⁻			
238	221	8	0	9	96.22%	92.86%	85.71%
183	155	13	0	15	91.80%	84.70%	69.40%
111	93	8	0	10	90.99%	83.78%	67.57%
158	129	11	0	18	88.61%	81.65%	63.29%
158	126	17	0	15	90.51%	79.75%	59.49%
204	162	23	0	19	90.69%	79.41%	58.82%
184	144	18	0	22	88.04%	78.26%	56.52%
195	150	24	0	21	89.23%	76.92%	53.85%
214	164	25	0	25	88.32%	76.64%	53.27%
206	147	34	0	25	87.86%	71.36%	42.72%
161	112	30	0	19	88.20%	69.57%	39.13%
240	149	32	0	59	75.42%	62.08%	24.17%
Average					**88.82%**	**78.08%**	**56.16%**
Standard deviation					**4.83%**	**7.91%**	**15.83%**

C

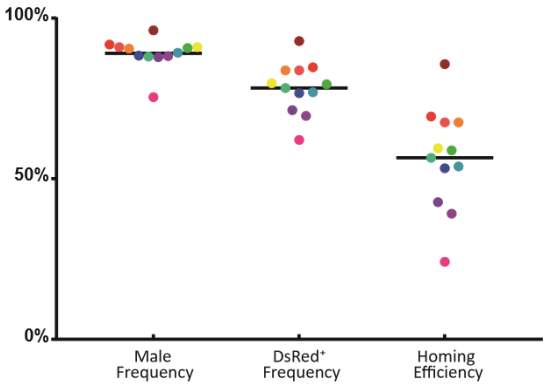
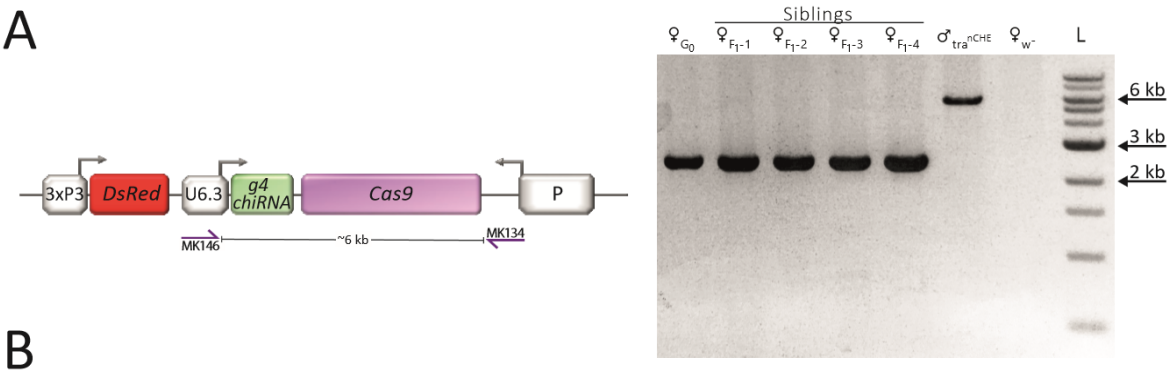


Fig. S3. Sex Conversion and Homing Efficiency.

(**A**) Mosaic intersex fly (right). Because of the high efficiency of Cas9 at cleaving wild-type *tra* alleles also in somatic cells, heterozygous tra^{nCHE}/+ XX individuals develop into infertile mosaic intersex individuals. These intersex individuals, often lack sex combs on one or both of their front legs, show a larger size, show a malformed ovipositor and have an inconsistent yellow and black patterns on their abdomen. (**B**) Estimation of the homing efficiency at the *tra* locus. Twelve tra^{nCHE}/+ males were crossed individually with virgin *w*– flies and progenies were screened for sex and presence of the DsRed eye marker. On average about 78% of the offspring carried the DsRed marker as opposed to the expected 50% based on Mendelian inheritance. The homing efficiency was calculated using the formula $\frac{DsRed^{+}-(Total/2)}{Total/2}$ and determined to be around 56%. (**C**) Dot plots showing the distribution of male frequency, DsRed-frequency, and homing efficiency values of the twelve replicates (colors represent the different replicates as indicated in panel B).

A



B

	M	K	M	D	A	D					S	S	G	T	Q	H	R	Indel
Tra	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	Size
1A-1	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
1A-2	ATG	AAA	ATG	GAT	GCC	---	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-6
1A-3	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
1A-4	ATG	AAA	ATG	GAT	GCC	GA-	-	A	ATG	GA	--	--T	GGA	ACC	CAG	CAT	CGA	0
2A-1	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
2A-2	ATG	AAA	ATG	GAT	GCC	GA-	-	T	GGA	T	GC	AGT	GGA	ACC	CAG	CAT	CGA	+3
2A-3	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
2A-4	ATG	AAA	ATG	GAT	G--	---	-				--	---	-GA	ACC	CAG	CAT	CGA	-12
4A-1	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
4A-2	ATG	AAA	ATG	GAT	GC-	---	-	A	TCC	CAT GC	--	---	--A	ACC	CAG	CAT	CGA	-3
4A-3	ATG	AAA	A--	---	---	---	-				--	---	---	-CC	CAG	CAT	CGA	-21
5A-1	ATG	AAA	ATG	GAT	GCC	GA-	-	T	GGA	T	GC	AGT	GGA	ACC	CAG	CAT	CGA	+3
5A-2	ATG	AAA	ATG	GAT	GCC	G--	-				GC	AGT	GGA	ACC	CAG	CAT	CGA	-3
5A-3	ATG	AAA	ATG	GAT	GCC	GA-	-				--	---	---	---	--G	CAT	CGA	-15
5A-4	ATG	AAA	ATG	GAT	GC-	---	-	A	TGG	ATG GA	--	--T	GGA	ACC	CAG	CAT	CGA	0
1B-1	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
1B-2	ATG	AAA	ATG	GAT	GCC	GAC	A				--	---	-GA	ACC	CAG	CAT	CGA	-6
2B-1	ATG	AAA	ATG	GAT	GCC	GAC	A	TC	CAC	T	GC	AGT	GGA	ACC	CAG	CAT	CGA	+6
2B-2	ATG	AAA	ATG	GAT	GCC	---	-		TGC	AA	--	--T	GGA	ACC	CAG	CAT	CGA	-3
3B-1	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
3B-2	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
4B-1	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
4B-2	ATG	AAA	ATG	GAT	GC-	---	-	A	T		--	---	---	-CC	CAG	CAT	CGA	-12
4B-3	ATG	AAA	ATG	GAT	GCC	---	-		AGC	ATC GAT GGA T	--	-GT	GGA	ACC	CAG	CAT	CGA	+6
4B-4	ATG	AAA	ATG	GAT	GCC	GA-	-	T	GC		--	--T	GGA	ACC	CAG	CAT	CGA	-3
5B-1	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
5B-2	ATG	AAA	ATG	GAT	GCC	G--	-	TG	GGT	GGA T	GC	AGT	GGA	ACC	CAG	CAT	CGA	+6
1C-1	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
1C-2	ATG	AAA	ATG	GA-	---	---	-	A			--	AGT	GGA	ACC	CAG	CAT	CGA	-9
2C-1	ATG	AAA	AT-	---	---	---	-	C	GAG		--	---	---	---	---	CAT	CGA	-21
2C-2	ATG	AAA	ATG	GAT	G--	---	-				--	---	-GA	ACC	CAG	CAT	CGA	-12
3C-1	ATG	AAA	ATG	GAT	GCC	GAC	A				GC	AGT	GGA	ACC	CAG	CAT	CGA	0#
3C-2	ATG	AAA	ATG	GAT	GCC	GAC	-		CCA	GCA T	--	---	---	-CC	CAG	CAT	CGA	-3
4C-1	ATG	AAA	ATG	GAT	GCC	---	-				--	---	---	---	CAG	CAT	CGA	-15
4C-2	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
5C-1	ATG	AAA	---	---	---	---	-				--	---	---	ACC	CAG	CAT	CGA	-21
5C-2	ATG	AAA	ATG	GAT	GCC	GAC	-				--	AGT	GGA	ACC	CAG	CAT	CGA	-3*
1D-1	ATG	AAA	ATG	GA-	---	---	-	A	CGG		--	---	---	ACC	CAG	CAT	CGA	-12
2D-1	ATG	AAA	ATG	GAT	---	---	-				--	---	GGA	ACC	CAG	CAT	CGA	-12
3D-1	ATG	AAA	ATG	GAT	---	---	-				--	---	GGA	ACC	CAG	CAT	CGA	-12
3D-2	ATG	AAA	ATG	GAT	G--	---	-	GA	ACC	CA	--	--T	GGA	ACC	CAG	CAT	CGA	-3
4D-1	ATG	AAA	ATG	GAT	GCC	---	-				--	---	---	---	CAG	CAT	CGA	-15
4D-2	ATG	AAA	ATG	GAT	GCC	GA-	-	T	GGA	TGC	--	AGT	GGA	ACC	CAG	CAT	CGA	+3
5D-1	ATG	AAA	ATG	GAT	G--	---	-	GG	CTC	CAG TTG GGG CAC TAC T	GC	AGT	GGA	ACC	CAG	CAT	CGA	+15
5D-2	ATG	AAA	ATG	GAT	GCC	GAC	A	TG	GAT	GTT CCG	--	---	---	ACC	CAG	CAT	CGA	+3

Fig. S4

Fig. S4. Example of aborted HDR and CHE-resistant *tra* alleles in generation F_6 of the population experiments.
(**A**) Aborted HDR: In one of the crosses of females with the DsRed eye marker no signs of an active drive system was observed. Amplification of the Cas9 expression cassette using *MK134/MK146* primer pair should result into a 6 Kb DNA fragment (Third lane from right; tra^{nCHE}). However PCR on the genomic DNA of the mother and its female offspring that carried the DsRed eye marker with these primers resulted in a truncated ~2.5 Kb product, indicating a large deletion in the CHE as a result of an aborted HDR event. (**B**) Molecular analysis of the CHE target site sequences in DsRed-marked females derived from all replicates (first number) of all four setting A–D (letter). The last number indicates different sequences from the same replicate. Few of the sequences of Setting A were obtained from F_{13}, as DsRed females were absent in F_6 of some replicates. The unchanged wild type tra allele was identified in six occasions (indel size: $0^{\#}$), but those individuals had defective tra^{nCHE} alleles with large deletions in the *Cas9* coding sequence (not shown) as the result of an aborted HDR (panel A). In all other DsRed females, tra^{Rst} mutations were found that represent in-frame indels, which have, at least, either the seed sequence of the target site or the CRISPR protospacer adjacent motif (PAM) abolished. The size of the indels were multiples of three keeping the *tra* coding sequence in-frame but destroying the g4 gRNA's recognition sequence at the cleavage site (indicated by a red triangle). The diversity of the indels indicates frequent independent events that can result in the emergence of resistant alleles. A three base pair AGC deletion at the site of cleavage was identified in eight independent occasions (-3*).

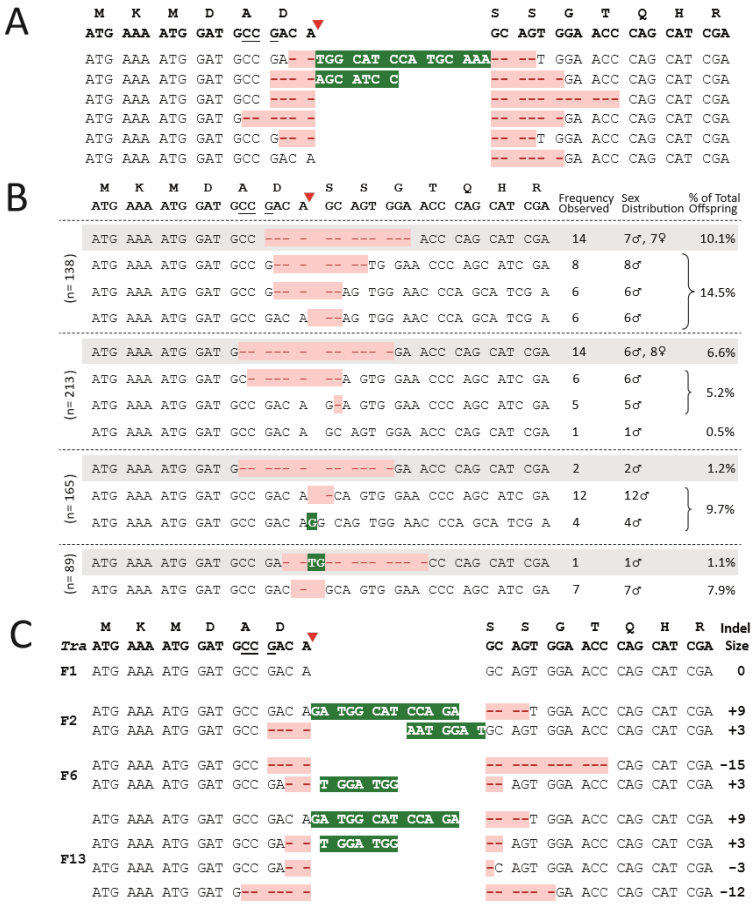


Fig. S5. Molecular analysis of non-HDR events at the site of cleavage.
(**A**) Molecular analysis of the CHE target site (red triangle indicates cleavage site) in F_1 female progeny of a cross between tra^{nCHE}/+ males and tra^{nDOCK}/+ virgins (Fig. 2) identified independent NHEJ events causing various in-frame indels (red/green) that resulted in resistant alleles. (**B**) Analysis of all ECFP/non-DsRed progeny derived from four individually crossed tra^{nCHE}/+ males and tra^{nDOCK}/+ virgins (separated by dashed lines) indicates the very efficient targeting of the *tra* locus in the presence of an active homing CHE (only one wild type allele in a progeny of 605). NHEJ-derived alleles were identified in 9–24% of all progeny causing frameshift mutations (tra^{-}) in about two thirds of these cases and in-frame indels (tra^{Rst}, shaded in grey) in about one third, whereby tra^{-} alleles were expectedly only observed in males or intersexes. The limited number of different NHEJ-derived alleles per single male cross indicates an activity at very early stages of primordial germ cell development. (**C**) Molecular analysis of the CHE target site (red triangle indicates cleavage position) in the *tra* locus of population experiment-derived DsRed-marked females. All sequences are taken from flies of the fourth replicate of setting D (*SI Appendix*, Fig. S4) across different generations. DsRed-marked females from F_1 carried a wild type tra allele but defective tra^{nCHE} alleles with large deletions in the *Cas9* coding sequence as the result of an aborted HDR (Fig. 2). In F_2 and F_6, various in-frame indel mutations were identified indicating independent emergences of these alleles. Some alleles already observed in F_2 and F_6 were also isolated in F_{13}, which implies the spread and fixation of these resistant alleles in the population.

A

Setting	No. of Replicates	No. of w^- Virgins	No. of w^- Males	No. of tra^{nCHE} Males
A (1:10)	5	30	30	3
B (1:2)	5	30	30	15
C (1:1)	5	30	30	30
D (1:0)	5	30	0	30

B

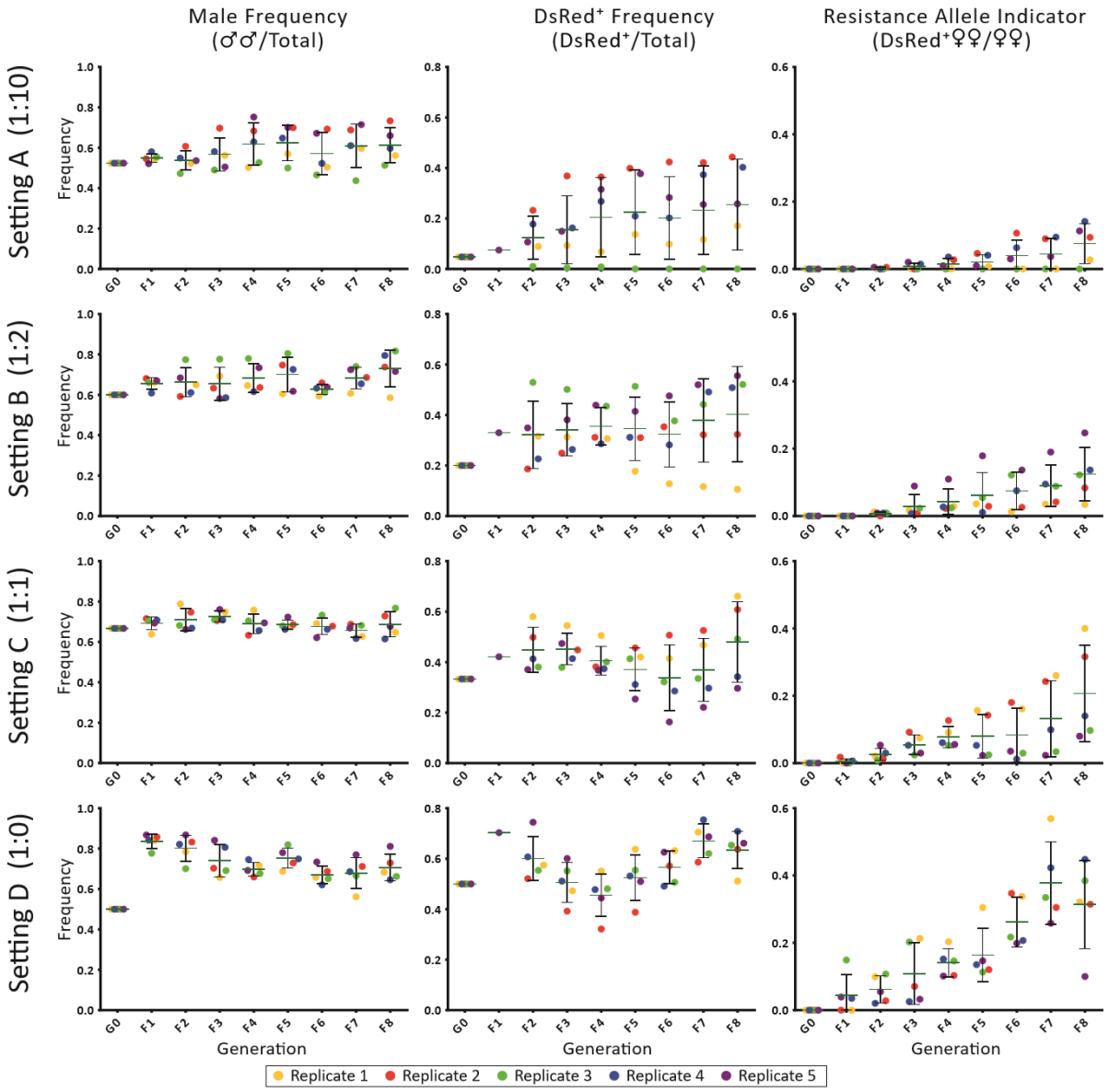


Fig. S6. Population experiments.

(**A**) Set-ups of the different cage experiments (settings A–D). Values in parentheses show the ratio of carrier tra^{nCHE} males to *w*– males used in each setting. (**B**) Dot plots representing the results of all five replicates (each indicated by different colors) for each setting of the population experiments. The increase in the DsRed females over the generations is an indicator for the emergence and spread of resistant alleles. Data for the DsRed frequency in F_1 is from replicate five only (purple dots). In setting A, replicate three (green dots) lost its tra^{nCHE} allele from F_2 onwards.

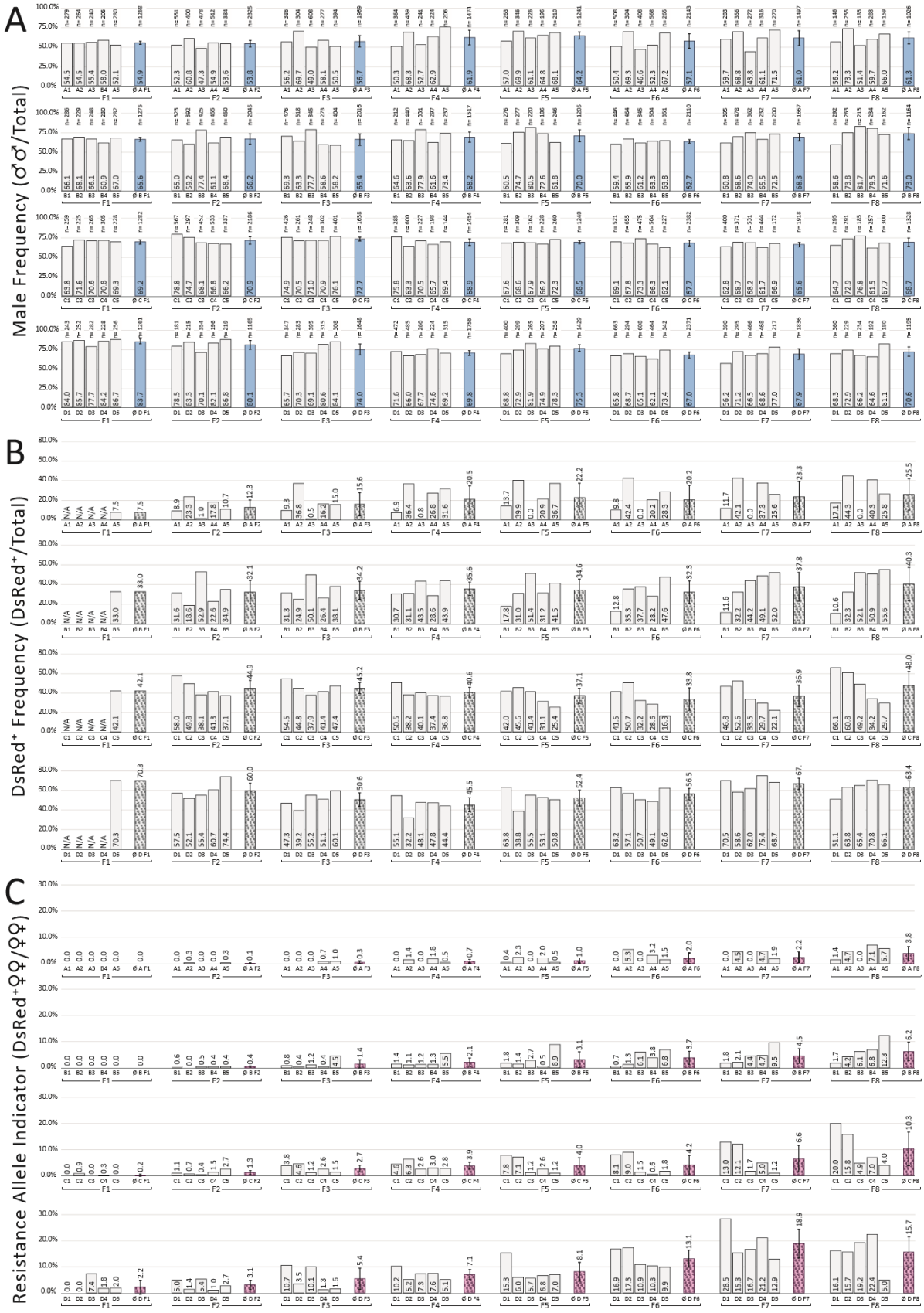

A
Male Frequency (♂♂/Total)
B
DsRed⁺ Frequency (DsRed⁺/Total)
C
Resistance Allele Indicator (DsRed⁺♀♀/♀♀)

Fig. S7.

Fig. S7. Original data of the population experiments.

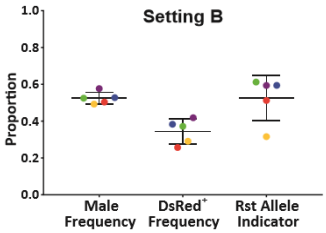
Five replicates for each of the four settings A–D (*SI Appendix*, Fig. S6*A*) were monitored over eight generations (F_1-F_8). (**A**) The frequency of males in the respective population. (**B**) The DsRed frequency depicting the percentage of flies carrying the DsRed eye marker (in F_1 data only available for replicate five). (**C**) The resistance allele indicator represented by the proportion of females that carry the DsRed eye marker. Color/pattern-marked columns represent mean and standard deviation for each setting and generation. These means were taken to produce Fig. 3 (please note, for the DsRed frequency in F_1, only replicate five was counted and that value taken as representative).

A

Setting B (1:2)

#	Total No. Flies	Male		Female		Male Frequency	DsRed$^+$ Frequency	Resistance Allele Indicator
		DsRed$^+$	DsRed$^-$	DsRed$^+$	DsRed$^-$			
1	337	98	68	54	117	49.26%	29.08%	31.58%
2	413	106	102	105	100	50.36%	25.67%	51.22%
3	347	129	53	101	64	52.45%	37.18%	61.21%
4	381	146	55	107	73	52.76%	38.32%	59.44%
5	454	190	72	114	78	57.71%	41.85%	59.38%
Average						**52.51%**	**34.42%**	**52.57%**
Standard Deviation						**3.25%**	**6.77%**	**12.36%**

B



C

Setting D (1:0)

#	Total No. Flies	Male		Female		Male Frequency	DsRed$^+$ Frequency	Resistance Allele Indicator
		DsRed$^+$	DsRed$^-$	DsRed$^+$	DsRed$^-$			
1	446	169	80	100	97	55.83%	37.89%	50.76%
2	327	121	62	77	67	55.96%	37.00%	53.47%
3	437	101	97	118	121	45.31%	23.11%	49.37%
4	432	109	99	113	111	48.15%	25.23%	50.45%
5	434	126	139	53	116	61.06%	29.03%	31.36%
Average						**53.26%**	**30.45%**	**47.08%**
Standard Deviation						**6.41%**	**6.73%**	**8.92%**

D

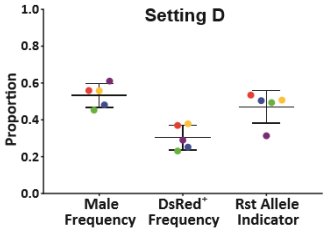


Fig. S8. Population collapse experiment data for generation F_{15}.

(**A**, **B**) Setting B and (**C**, **D**) Setting D populations recovered from the masculinizing effect of the *tra*^{nCHE} homing allele as the sex ratios are back to around normal 50%. The DsRed eye marker was present in about one third of the population but in almost half of the females serving as resistance allele indicator. Original data (**A**, **C**) and dot plot representation (**B**, **D**) of all five replicates (each indicated by a different color). The averages were taken to produce Fig. 3.

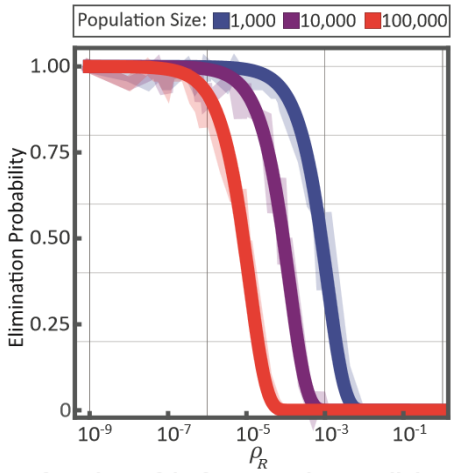


Fig. S9. Elimination probability as a function of in-frame resistant allele generation rate, ρ_R, for a range of population sizes, *N*, between 1,000 and 100,000.

We consider the scenario in which *tra*^{nCHE}; XX individuals are fertile males, and assume a 1:1 release to wild ratio, that CRISPR-mediated cleavage efficiency is 100%, that the probability of accurate homology-directed repair following cleavage is 90%, that the in-frame resistant allele generation rate is as specified above, that in-frame resistant alleles have no associated fitness cost, and that the remainder of resistant alleles are out-of-frame or other mutations. Sigmoidal curves are fitted to data points covering 30 in-frame resistant allele generation rates sampled logarithmically between 10^{-1} and 10^{-9}. Faint lines represent interpolation between simulated data points while solid lines represent fitted sigmoidal relationships. For an adult population size of 1,000, an in-frame resistant allele generation rate of less than 10^{-4} is required to achieve likely elimination, while for an adult population size of 100,000, an in-frame resistant allele generation rate of less than 10^{-6} is required to achieve likely elimination.

Male \ Female	DR/XX	RR/XX	RB/XX	WR/XX	WB/XX	WW/XX
DD/XY	(1/2) DR (1/2) DD	(1) DR	(1/2) DR (1/2) DB	(1/2) WD (1/2) DR	(1/2) WD (1/2) DB	(1) WD
WD/XY	((1+e)/4) DD ((1+e+ρ_R)/4) DR (ρ_R/4) RR (ρ_B/4) DB (ρ_B/4) RB	((1+e)/2) DR (ρ_R/2) RR (ρ_B/2) RB	((1+e)/4) DR ((1+e)/4) DB (ρ_R/4) RR ((ρ_R+ρ_B)/4) RB (ρ_B/4) BB	((1+e)/4) WD ((1+e)/4) DR (ρ_R/4) WR (ρ_R/4) RR (ρ_B/4) WB (ρ_B/4) RB	((1+e)/4) WD ((1+e)/4) DB (ρ_R/4) WR (ρ_R/4) RB (ρ_B/4) WB (ρ_B/4) BB	((1+e)/2) WD (ρ_R/2) WR (ρ_B/2) WB
DR/XY	(1/4) DD (1/2) DR (1/4) RR	(1/2) DR (1/2) RR	(1/4) DR (1/4) DB (1/4) RR (1/4) RB	(1/4) WD (1/4) WR (1/4) DR (1/4) RR	(1/4) WD (1/4) WR (1/4) DB (1/4) RB	(1/2) WD (1/2) WR
DB/XY	(1/4) DD (1/4) DR (1/4) DB (1/4) RB	(1/2) DR (1/2) BR	(1/4) DR (1/4) DB (1/4) RB (1/4) BB	(1/4) WD (1/4) WB (1/4) DR (1/4) RB	(1/4) WD (1/4) WB (1/4) DB (1/4) BB	(1/2) WD (1/2) WB
RR/XY	(1/2) DR (1/2) RR	(1) RR	(1/2) RB (1/2) RR	(1/2) WR (1/2) RR	(1/2) WR (1/2) RB	(1) WR
RB/XY	(1/4) DR (1/4) RR (1/4) DB (1/4) RB	(1/2) RR (1/2) RB	(1/4) RR (1/2) RB (1/4) BB	(1/4) WR (1/4) WB (1/4) RR (1/4) RB	(1/4) WR (1/4) WB (1/4) RB (1/4) BB	(1/2) WR (1/2) WB
BB/XY	(1/2) DB (1/2) RB	(1) RB	(1/2) RB (1/2) BB	(1/2) WB (1/2) RB	(1/2) WB (1/2) BB	(1) WB
WR/XY	(1/4) WD (1/4) WR (1/4) DR (1/4) RR	(1/2) WR (1/2) RR	(1/4) WR (1/4) WB (1/4) RR (1/4) RB	(1/4) WW (1/2) WR (1/4) RR	(1/4) WW (1/4) WR (1/4) RB (1/4) WB	(1/2) WW (1/2) WR
WB/XY	(1/4) WD (1/4) WR (1/4) DB (1/4) RB	(1/2) WR (1/2) RB	(1/4) WR (1/4) WB (1/4) RB (1/4) BB	(1/4) WW (1/4) WR (1/4) WB (1/4) RB	(1/4) WW (1/2) WB (1/4) BB	(1/2) WW (1/2) WB
WW/XY	(1/2) WD (1/2) WR	(1) WR	(1/2) WR (1/2) WB	(1/2) WR (1/2) WW	(1/2) WB (1/2) WW	(1) WW
DD/XX	(1/2) DR/XX (1/2) DD/XX	(1) DR/XX	(1/2) DR/XX (1/2) DB/XX	(1/2) WD/XX (1/2) DR/XX	(1/2) WD/XX (1/2) DB/XX	(1) WD/XX
WD/XX	((1+e)/4) DD/XX ((1+e+ρ_R)/4) DR/XX (ρ_R/4) RR/XX (ρ_B/4) DB/XX (ρ_B/4) RB/XX	((1+e)/2) DR/XX (ρ_R/2) RR/XX (ρ_B/2) RB/XX	((1+e)/4) DR/XX ((1+e)/4) DB/XX (ρ_R/4) RR/XX ((ρ_R+ρ_B)/4) RB/XX (ρ_B/4) BB/XX	((1+e)/4) WD/XX ((1+e)/4) DR/XX (ρ_R/4) WR/XX (ρ_R/4) RR/XX (ρ_B/4) WB/XX (ρ_B/4) RB/XX	((1+e)/4) WD/XX ((1+e)/4) DB/XX (ρ_R/4) WR/XX (ρ_R/4) RB/XX (ρ_B/4) WB/XX (ρ_B/4) BB/XX	((1+e)/2) WD/XX (ρ_R/2) WR/XX (ρ_B/2) WB/XX
DB/XX	(1/4) DD/XX (1/4) DR/XX (1/4) DB/XX (1/4) RB/XX	(1/2) DR/XX (1/2) BR/XX	(1/4) DR/XX (1/4) DB/XX (1/4) RB/XX (1/4) BB/XX	(1/4) WD/XX (1/4) WB/XX (1/4) DR/XX (1/4) RB/XX	(1/4) WD/XX (1/4) WB/XX (1/4) DB/XX (1/4) BB/XX	(1/2) WD/XX (1/2) WB/XX
BB/XX	(1/2) DB/XX (1/2) RB/XX	(1) RB/XX	(1/2) RB/XX (1/2) BB/XX	(1/2) WB/XX (1/2) RB/XX	(1/2) WB/XX (1/2) BB/XX	(1) WB/XX

Fig. S10. Crosses representing the inheritance pattern of a CRISPR-based homing system targeting the *tra* locus in *C. capitata*.

"D" denotes the drive allele, tra^{nCHE}, "W" denotes the wild-type *tra* allele, "R" denotes a drive-resistant allele with an in-frame internal deletion at the *tra* locus, tra^{Rst} and "B" denotes a drive-resistant null allele tra^{-}. *C. capitata* is an XY species in which female development requires presence of the *tra* allele, hence XY individuals are fertile males, XX individuals with a functioning *tra* allele (i.e. having the genotypes WW, WR, WB, RR, DR and RB) are fertile females, and XX individuals without a functioning *tra* allele (i.e. having the genotypes DD, DB and BB) are fertile males. The only exception is WD/XX individuals, which are infertile intersex individuals, unless a uniform somatic destruction of the *tra* locus in all cells is guaranteed by using multiple guide RNAs and an early embryogenic promoter such as *Sry-α* promoter. Homing is only manifest in WD heterozygotes, whereby WD individuals produce D gametes in the germline at a frequency equal to (1+*e*)/2, where *e* denotes the proportion of W gametes that are converted into D gametes through the act of homing. Homing-resistant alleles may be generated during the process of DNA cleavage and repair whereby WD individuals produce resistant alleles that are in-frame indels, R, at a rate equal to $\rho_R/2$, and produce cleavage resistant alleles that are out-of-frame or other mutations, B, at a rate equal to $\rho_B/2$. Crosses involving WD/XX males are shaded out as WD/XX individuals may be rendered either infertile intersex or fertile males as described above. Offspring are half XX and half XY. The inheritance pattern of the homing and resistant alleles depicted here is incorporated into the population dynamic model described above and in *SI Appendix*, Fig. S11.

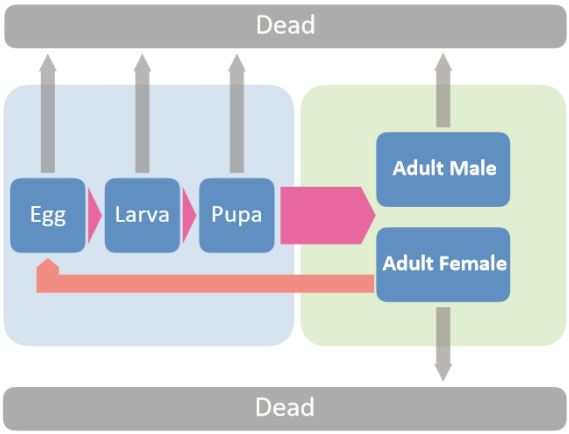


Fig. S11. Population dynamic model of *C. capitata.*
Eggs develop into larvae which develop into pupae, some of which develop into adult males and some into adult females. Death can occur at any life stage, and adult females lay eggs following fertilization. Additional density-dependent mortality occurs at the larval stage. Parameter values are provided in *SI Appendix*, Table S1, and the equations describing the equivalent implementation of this model for *An. gambiae* are included in Supplementary File S1 of Marshall *et al.*, 2017 (18).

SI Tables

Table S1. Parameter values for population genetic/ dynamic model for *C. capitata.*

Symbol:	Parameter:	Value:	References:
Primary parameters:			
β	Egg production per adult female	20 /day	Diamantidis *et al.*, 2011 (21)
T_E	Duration of egg stage	2 days	Diamantidis *et al.*, 2011 (21)
T_L	Duration of larval stage	6 days	Diamantidis *et al.*, 2011 (21)
T_P	Duration of pupal stage	10 days	Diamantidis *et al.*, 2011 (21)
μ_M	Death rate of adult stage	0.1 /day	Carey *et al.*, 2005 (22)
r_M	Population growth rate (in absence of density-dependent mortality)	1.031 /day	Nyamukondiwa *et al.*, 1980 (20)
c	Probability of CRISPR-mediated cleavage in WD heterozygote	1.0	Champer *et al.*, 2017 (19)
P_{HDR}	Probability of accurate homology-directed repair given cleavage	0.90	This paper
Variable parameters:			
ϑ	Proportion of resistant alleles that are in-frame internal deletions	[1/3, (1/3) x 10^{-4}]	This paper
N	Equilibrium adult medfly population size (male and female)	[10^3, 10^6]	Diamantidis *et al.*, 2011 (21)

Table S2. List of primers used in this study.

ID	Name	Sequence
MK024	DTra_F	CGGCGACAAGCTTGAGGTACCCACTATATAGTATAAC
MK025	DTra_R	CTATAGGGCGAATTGTGTAGCCAAATCGCGGAACTC
MK037	G1-S	CTTCAGCAGTGGAACCCAGCATCG
MK038	G1-AS	AAACCGATGCTGGGTTCCACTGCT
MK041	G3-S	CTTCGATCGGTTATACTATATAGT
MK042	G3-AS	AAACACTATATAGTATAACCGATC
MK043	G4-S	CTTCGTGCTGGGTTCCACTGCTGT
MK044	G4-AS	AAACACAGCAGTGGAACCCAGCAC
MK048	G5-S	CTTCTATAGTGGGTACCTCGATGC
MK049	G5-AS	AAACGCATCGAGGTACCCACTATA
MK058	Tra_T7endo_F	CCTGCTAATTCTGCTTTCCCTATGTTTGTG
MK059	Tra_T7endo_R	CCTCGTCTGCAAAGTACGGAATCTTGTG
MK060	attB-SmaI-S	CTAGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCG CACCGCGG
MK061	attB-SmaI-AS	AGCTCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCG CACCGCGG
MK072	aTubE1_F	CCAAGTGACCGCGGATCTTCATATTCGTTTTACGTTTGTCAAGCCTC
MK073	aTubE1_R	TCGTGGTCCTTATAGTCCATATTGAGTTTTTATTGGAAGTGTTTCAC
MK075	aTubI1GT_R	TCGTGGTCCTTATAGTCCTCAACCTGTGGATGAGGAGGAAGGGAAAACGGATG
MK076	Cas9DYK_F	GACTATAAGGACCACGACGGAGACTACAAGGATCATG
MK077	Cas9_R	GATCTAGATCTGCGGCCGATCACTAGATTACTTTTTCTTTTTTGCCTG
MK078	HMA_F1	CGGTCACACTGAGGAAAGTG
MK079	HMA_R1.1	CAACAAAAAGATGGCACTGG
MK085	Cas9_SeqR1	TGGTGCTCGTCGTATCTC
MK086	Cas9_SeqR2	TTGATAATTTTCAGCAGATCGTG
MK087	Cas9_SeqR3	CTTGTTGTCGATGGAGTC
MK088	Cas9_SeqR4	CAGCACAGAATAGGCCAC
MK116	3xP3attP_F	ACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGGGGATTATTCATTAGAGAC
MK117	SV40attP_R	GGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGATGATGAGTTTGGACAAACCAC
MK122	Transformer4_UR	CAAAGGTTACCCCAGTTGGGGCACTACTCTGTCGGCATCCATTTTCATC
MK123	Transformer4_DF	CTCAAAGGTTACCCCAGTTGGGGCACTACTGCAGTGGAACCCAGCATCGAG
MK126	Tra_HRCheck_F	CCGACCGAATCGTGAGGACTTGAAG
MK127	Tra_HRCheck_R	GAATTAAGTAACTTCCACTTCCTAACTCGTGTGAC
MK128	XFPct_F	AACGAGAAGCGCGATCACATGGTC
MK129	XFPnt_R	ACGCTGAACTTGTGGCCGTTTACGTC
MK134	attBaTubE1_F	GGGCGCGTACTCCACCTCACCTAGGTGACTCGAGTTCATATTCGTTTTACGTTTGTC
MK135	Cas_attBSmaI_AscIR	GGGCGCGTACTCCACCTCACGGCGCGCCATTACTTTTTCTTTTTTGCCTG
MK138	DPa180_AvrIIF	AATAACCTAGGTGGTGATCATTGTTCTTTCTTACTTGGTG
MK139	DPa180_XhoIR	TAATCCTCGAGTAATAATTTCCCCGTGTTGTGCTG
MK140	Rcd1r_XbaIF	AATAATCTAGACACGGCCAAATCGATGCAGAC
MK141	Rcd1r_XhoIR	TAATCCTCGAGGTTAGCTTGCAAAGATCTAGTAG
MK142	Srya_AvrIIF	AATAACCTAGGGCCACCAGCAGTTCAAGACCAAG
MK143	Srya_XhoIR	TAATCCTCGAGTATCAGATGTGCTCCGGGAAACAG
MK144	bTub3UTR_F	AAAAGTAATGGCGCGATTAACTTCCCACTCAAGATCAC
MK145	bTub3UTR_R	CGCTTAATGCGTATGGTTTAGGTTTATGCAATGCCT
MK146	U63P_F	CTGTTTTGCTCACCTGTGATTGCTCCTACTC
MK147	U63DS_R	CATACGCATTAAGCGAACATTAAAAAGATG
MK148	3xP3attB_F	CCACCTCACGGCGCGGGGGATTATTCATTAGAGAC
MK149	SV40toU63_R	AGGTGAGCAAAACAGGATGATGAGTTTGGACAAACCAC
MK153	PhiC31_BsaI_F	ATGGTCTCACATGGACACGTACGCGGGTGCTTACGAC
MK154	PhiC31_NotI_R	GTGTATGCGGCCGCTTACTAGGCAGCTACGTCTTC
MK155	HRMA_OT1F	GGACCAGGAGCGTTATCTG
MK156	HRMA_OT1R	GGCAAATTGATGTCGAGCAC
MK157	HRMA_OT2F	CCATATCCGACCTGACCAC
MK158	HRMA_OT2R	CGGTTGCTGTTCCGTTTC
MK159	HRMA_OT3F	CAGCTTGTTGTCCTCGATG
MK160	HRMA_OT3R	GTGGCAGACCGAATCCAG

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4 Discussion

CRISPR/Cas-based methods for genome editing have revolutionized the biological sciences more than any other known technologies thus far. As described in detail in sections 2.3.5, and 2.4.2.2 the uncomplicated guide RNA-mediated programmability of the Cas9 endonuclease for the introduction of DSBs at a precise genomic position has greatly simplified all aspects of genome editing and, moreover, made it feasible to design novel reproductive sterility and sexing approaches for transgenic SIT (chapter 3.2.2 & 4.1; Eckermann et al., 2014; Kandul et al., 2019), as well as to engineer synthetic homing endonuclease genes (HEGs) that can be leveraged for use as homing CRISPR/Cas gene drives (HCGD) in genetic insect control (see 2.3.5, 3.3, 4.3 & 4.4; Esvelt et al., 2014; KaramiNejadRanjbar et al., 2018). While holding great promises, most of these novel approaches are still in their early stages of development, leaving a number of remaining obstacles and shortcoming that will need to be overcome to increase efficiency, enhance genetic stability, and enable their confined application in order meet ecological concerns necessary to set grounds for a legal framework and international agreements for future releases. Against this background, the following chapter will discuss recently published alternative applications of the CRISPR/Cas technology for the enhancement of transgenic SIT, as well as highlighting novel improvements and innovations of existing genome editing and gene drive approaches.

4.1 Improved sexing and reproductive sterility by transgenic CIRSPR/Cas9-based approaches: precision guided SIT

With the exception of the killing-sperm approaches currently under development (results sections 3.2.1 & 3.2.3), most of the present transgenic SIT reproductive sterility and sexing approaches are based on the generation of insects that possess and pass on an effector transgene, whose ectopic expression in the early embryo causes lethality in either all descendants (sterility system) or only the female offspring (sexing system) (Fu et al., 2007; Gong et al., 2005; Horn and Wimmer, 2003; Ogaugwu et al., 2013; Schetelig et al., 2009a; Thomas et al., 2000; chapters 2.3.2,*i* & 2.3.2,*ii*). However, the CRISPR/Cas technology (2.4.2.2, *ii*) has enabled researchers to diversify the design of transgenic SIT approaches by exploiting its capability to target, cleave and thereby induce loss-of-function mutations in essential genes (e.g. essential for female sex determination, fertility, or viability) or even destroy entire chromosomes (Eckermann et al., 2014; Galizi et al., 2016). One approach of how this capability could be harnessed is elaborated in the results section 3.2.2, where we propose to express Cas9 specifically during

spermatogenesis together with an array of guide RNAs that route the endonuclease to several target sites, which occur repetitively and on all chromosomes. This should consequently result in a multitude of DSBs, which will ultimately lead to chromosome shredding in the sperm, entailing a multifactorial male reproductive sterility that is similar to the outcome of irradiation-based sterilization of classical SIT (Eckermann et al., 2014).

More recently, a novel strategy named precision guided SIT (pgSIT) has been developed as proof-of-concept in *D. melanogaster*, which employs the CRISPR/Cas technology, to produce flies carrying a double-knockout of two essential genes that are vital for female survival and male fertility, respectively, permitting simultaneous sex-separation and male sterilization (Kandul et al., 2019). To this end, two independent strains were generated: A ubiquitously Cas9 protein expressing strain (*Ubi-Cas9*), and a strain expressing a multiplexed double guide RNA (*dgRNA*), targeting a female-specific exon of the sex-determination gene *sex lethal* (*Sxl*) and the spermatogenesis-specific gene *βTubulin 85D* (*βTub*). Crossing females of the *Ubi-Cas9* strain and males of the $dgRNA^{\beta Tub,Sxl}$ strain gives rise to developing embryos, in which Cas9-induced DSBs are being steadily produced at the respective target sites in *Sxl* and *βTub*. The DSBs are subsequently mended by the non-homologous end joining (NHEJ) DNA repair pathway, resulting in a mosaic of deleterious insertions-deletions mutations (indels), which consequently lead to knockout alleles of *Sxl* and *βTub*. Although in some cells the *Sxl* and/or *βTub* loci are not targeted, and thus remain wild-type alleles, female lethality and male sterility is still achieved in 100% of the respective progeny, as the critical thresholds that determine the manifestation of the knockout phenotypes is always surpassed – a phenomena the authors refer to as "lethal biallelic mosaicism". In conformity with the in section 2.3.2,*ii* mentioned ensuing advantage when releasing larvae instead of adult individuals to intentionally evoke resource competition with larvae of the wild population (Atkinson et al., 2007; Phuc et al., 2007), sgSIT would be particularly suitable for biocontrol of mosquito species, as females predominantly survive until pupal transition and the method enables the release of diapausing embryos. However, the present pgSIT approach will require further elaboration with regard to the practical aspect of mass rearing, as it is based on the cross of two homozygous strains immediately before the release generation, which is not practicable, especially since currently methods to separate virgin females and males from the homozygous *Ubi-Cas9* or $dgRNA^{\beta Tub,\ Sxl}$ strains are lacking. Moreover, *βTub* is not the best gene to target for reproductive sterility, as it will cause immobile sperm, which will not be efficiently transferred and used for insemination events.

4.2 Strategies to increase HDR rates in genome editing

A major restraint of HDR-based genome editing (2.4.2.2) and homing-based gene drives (2.3.5 & 3.3) in metazoans is that HDR rates are usually found to be rather low compared to the much more frequently occurring NHEJ events (Carroll, 2014; Harrison et al., 2014; Kane et al., 2016). While HDR is the prevailing DSB repair pathway in baker's yeast, *Saccharomyces cerevisiae* (Clikeman et al., 2001; González-Barrera et al., 2003; Haber, 1995; Pâques and Haber, 1999), there are only a few described exceptional species- and cell type-specific examples in multicellular eukaryotes that exhibit generally elevated HDR rates, such as chicken B lymphocytes, mouse embryonic stem cells, and mouse neural precursor cells (Shrivastav et al., 2008). However, in the vast majority of metazoans, HDR is largely restricted to late S phases, and G2 phases of dividing cells (Hartlerode et al., 2011; Lieber et al., 2003), which is considered a natural safety mechanism for two reasons: Firstly, during these phases a sister chromatid (mitosis), or homologous chromosome and sister-chromatid (meiosis) is present in close proximity that can serve as a secure homologous repair template (Orthwein et al., 2015), and secondly, it avoids telomere fusion during M and early G1 phases (Lin et al., 2014; Orthwein et al., 2014). In the mouse, HDR even seems to be restricted exclusively to the female germline (Grunwald et al., 2019). In contrast, NHEJ is enabled throughout all cell cycle stages (Lieber et al., 2003), which imparts this repair pathway with a dominant role in metazoan DSB repair (Carroll, 2014), and in turn limits the efficiency of HDR-based genome editing approaches.

Therefore, several counter-measures have been undertaken to enhance the rates of HDR (Kane et al., 2016). One type of strategy aims at either the suppression of the NHEJ pathway through gene knock-out (Beumer et al., 2008; Bozas et al., 2009; Ma et al., 2014; Morton et al., 2006), RNAi-based downregulation (Basu et al., 2015), or small molecule-mediated inhibition (G. Li et al., 2017; Lisowski, 2018; Maruyama et al., 2015) of essential NHEJ pathway components, such as *Lig4* or *KU70* (Carroll and Beumer, 2014; Chu et al., 2015), or, *vice versa*, at the upregulation of genes that encode for key players of the HDR pathway, including *RAD51* (Jayathilaka et al., 2008; Klovstad et al., 2008; Orthwein et al., 2015; Song et al., 2016; Yu et al., 2015). Other approaches to augment HDR focus on the use of chemical compounds that arrest cells at the G2 phase (Lin et al., 2014), and yet others concentrate on the optimization of the donor repair template (Beumer et al., 2013; Miura et al., 2015; Richardson et al., 2016). However, the actual effectiveness and broad applicability of several of these measures remains controversial, as, for example, some chemical compounds have been found to be cytotoxic or enhance HDR only in a cell- and/or species-specific manner (Ye et al., 2018). Furthermore, permanent *lig4* knock-out is only tolerated in some species, but causes lethality in others (Basu et al., 2015; Frank et al., 2000, 1998), and was shown to fail to promote HDR when only knocked-down transiently (Basu et al., 2015;

Häcker and Schetelig, 2018). In contrast, elaborations regarding donor repair template design – e.g. regarding the length of homology arms, or whether to provide it in form of DNA plasmids or as linear dsDNA or ssDNA, are considerations that seems to be of more general applicability (Richardson et al., 2016; Yang et al., 2014).

Moreover, and besides these HDR-specific measures, a foremost factor for precise and successful genome editing approaches, which indirectly also increases the HDR rates, is the general accuracy and performance of the endonuclease itself to efficiently generate DSBs at the desired genomic locus. In this regard, considerable attention has been given to the careful selection of suitable promoters/enhancers and 3'UTRs for a timely and efficient expression and translation (e.g. germline specific) of the programmable endonuclease (Chan et al., 2013a; Port et al., 2014; Song and Stieger, 2017; Zhang et al., 2017) as well as guide RNAs (Port et al., 2014; Port and Bullock, 2016). In addition, different formats, in which the CIRSPR/Cas9 components can be delivered into cells have been established, including their introduction as DNA expression plasmids (Gratz et al., 2014; Kouranova et al., 2016; Port et al., 2014), *in vitro* transcribed gRNA(s) and *cas9* mRNA (Yang et al., 2014), recombinant Cas9 protein (Lee et al., 2014), or through the expression of Cas9 in transgenic strains (Gratz et al., 2014; Kondo and Ueda, 2013; Port et al., 2014; Ren et al., 2013; Sebo et al., 2014). The use of pre-assembled ribonucleoproteins (RNPs) complexes, consisting of recombinant Cas9 protein and *in vitro* transcribed guide RNA, has proven to be particularly effective for the generation of DSBs, as it bypasses the delay time necessary for transcription and/or translation (Kim et al., 2014). Application of RNPs has already been successfully applied in several agricultural pests and disease vector insect species, including *Ceratitis capitata* (Meccariello et al., 2017), *Drosophila suzukii* (Ahmed et al., 2019; Kalajdzic and Schetelig, 2017), and *Aedes aegypti* (Basu et al., 2015; Chaverra-Rodriguez et al., 2018; Kistler et al., 2015). Besides, Kim et al. have reported that using RNPs can reduce the incidence of off-target events due to the rapid degradation of the complex (Kim et al., 2014). Lastly, a tremendous number of *cas9* orthologs (Esvelt et al., 2013; Hirano et al., 2016; Ran et al., 2015; Xu et al., 2015, p. 201; Zetsche et al., 2015; Zhang et al., 2013) and variants (Anders et al., 2016; Kleinstiver et al., 2016, 2015b, 2015a; Slaymaker et al., 2016) have been identified and engineered, respectively, which differ, for example, in their overall fidelity, PAM specificity, requirement for a tracrRNA, and also the kind of DSB they generate (blunt or staggered DNA ends), which could be applied if the standard Cas9 of *S. pyogenes* is unsuitable, e.g. due to the lack of a PAM sequence near the desired target site.

4.3 Improvements and alternatives to homing CRISPR/Cas9 gene drives for avoidance of resistance allele formation

Homing endonuclease genes (HEGs) can increase their copy number in the genome by copying themselves via cleavage-induced HDR to the respective position on the homologous chromosome. The idea to recreate this overreplication-based gene drive mechanisms with engineered HEGs to enable super-Mendelian inheritance that can be harnessed to spread a genetic trait into a population was first proposed by Austin Burt in 2003 (Burt, 2003). However, as described in detail in chapter 2.3.5, reconfiguration of the target sequence of natural HEGs, or utilization of ZFNs or TALENs has been found to be impracticable (Chan et al., 2013a), wherefore it was only with the advent of the CRISPR/Cas technology that it became workable to generate artificial homing elements. The fundamental components of such a CRISPR/Cas9 homing element (CHE) include the Cas9 encoding gene under control of a germline-specific promoter and a guide RNA (gRNA) designed to direct Cas9 to the corresponding wild-type allele on the homologous chromosome. Upon Cas9-mediated DNA cleavage, the DSB will ideally be mended by HDR for which the CHE-bearing allele will be used as repair template, converting wild-type alleles to drive alleles, and hence heterozygotes to homozygotes. Since its theoretical conceptualization (Esvelt et al., 2014), several HCGDs have been suggested and executed that are either designed to cause a population suppression by targeting and, thereby, disrupting a critical gene, with consequences such as recessive lethality, female sterility or female to male sex-conversion (Hammond et al., 2016; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Simoni et al., 2020), or to prompt a population replacement in which a gene required e.g. for pathogen replication or transmission is destroyed. Alternatively, CHEs for use in replacement drives can also be equipped with a cargo, or, so-called payload genes, that e.g. encode for an antimalarial antibody (Champer et al., 2016; Gantz et al., 2015).

However, a major inherent problem of current HCGDs is that the induced DSBs are frequently repaired by NHEJ instead of HDR (4.2), which often leads to short insertion and deletions (indels) at the guide RNA recognition site, resulting in the formation of cleavage resistant alleles (Champer et al., 2017; Hammond et al., 2017; KaramiNejadRanjbar et al., 2018; Marshall et al., 2017; Unckless et al., 2017). While for replacement HCGDs any cleavage resistance mutation will contribute to impede the spread of the CHE, the direct impact of an NHEJ-generated indel mutation for a suppression HCGD depends on whether the mutation results in the loss or preservation of the target gene function – as the combination of a CHE allele and an indel-based loss of function allele will still lead to sterility, infertility or sex-conversion and will therefore not be further inherited. However, as we revealed in our in-dept analysis of resistance allele formation in a *transformer*-targeting female to male sex-conversion homing suppression gene drive in *D. melanogaster* (chapter 3.3), NHEJ-based DSB-repair readily creates small in-frame indel mutations

that retain *tra* function, which consequently allows unimpeded female development of XX individuals instead of developing into infertile male intersexes (KaramiNejadRanjbar et al., 2018). Due to the high fitness costs that the drive implicates on females and the associated strong selective pressure on the *tra* locus for the survival of the population, such in-frame drive-resistant alleles evolve constantly at a fast rate, are inherited, and quickly accumulate, which results in a complete halt of the drive, leading to a population rebound after some, as in our case 15 generations (KaramiNejadRanjbar et al., 2018).

From these results we have concluded, in accordance with other laboratories, that an extension of the current CHE-construct architecture by employing multiple gRNAs that target the respective gene at several positions, could significantly reduce the development of homing-resistant alleles, as it is unlikely that NHEJ-based resistance mutations will be generated at all sites simultaneously (Champer et al., 2017; Hammond et al., 2017; KaramiNejadRanjbar et al., 2018; Marshall et al., 2017). This conception was further underpinned by a mathematical framework, in which we modeled our obtained data in the context of a multiplex scenario (KaramiNejadRanjbar et al., 2018), as well as by two concurrently conducted empirical studies in *D. melanogaster*, that have employed two (Champer et al., 2018) or four (Oberhofer et al., 2018) gRNAs in each of their CHE-designs, targeting the non-essential *white* or *cinnabar* gene, or the recessive sterility genes *yellow-g* or *deformed*, respectively. An accessory advantage arising from the use of multiple gRNAs is that successful homing is likely to take place even in the presences of pre-existing or naturally emerging genetic variations at the target site in a population (Champer et al., 2018; Marshall et al., 2017; Oberhofer et al., 2018). Additionally, both studies point out that resistance allele formation can further be decreased by the careful selection of an appropriate promoter that restricts *cas9* expression and, thus, cleavage-activity to the germline, which possess elevated HDR-rates, and avoid parental carryover of the Cas9-*gRNA* ribonucleoprotein into the zygote, where NHEJ-based repair is prevailingly active (4.2). However, while the studies demonstrated that multiplexing approaches can effectively decrease the origination of drive resistance alleles, Champer and colleagues identified a possible weak point that arises from the scenario when the two outermost gRNA target sites are cleaved simultaneously, as this, when repaired via NHEJ, will lead to a larger deletion and the loss of all target sites at once (Champer et al., 2018). On the same grounds as stated above, this should be, yet again, an issue largely restricted to replacement homing strategies.

In about the same period, Kyrou et al. developed an elegant alternative strategy to the multiplex-based approaches in order to counteract resistance allele formation in a Cas9-based sterility suppression gene drive in *An. gambiae*, which requires only a single gRNA (Kyrou et al., 2018). This refinement of previous approaches is based on the careful selection of the gRNA target site for a highly conserved intragenic sequence that does not tolerate any variation for the maintenance of its functionality. Following this concept, the authors have opted for the *doublesex* (*dsx*) gene, whose male- and female- specific

isoforms are produced by sex-specific alternative splicing that play a pivotal role in the sex-determination pathway of many insects (Saccone et al., 2002). In *An. gambiae* only the female-specific splice variant contains the exon 5 (Scali et al., 2005), which is highly conserved among species of the *An. gambiae* complex and is vital for female development, since XX individuals lacking this exon develop a sterile intersex phenotype (Kyrou et al., 2018). Taking advantage of this, Kyrou et al. designed a CHE comprising *cas9* under the control of the *zero population growth* (*zpg*) germline-specific promoter in combination with a gRNA targeting the splice acceptor site at the intron 4 – exon 5 boundary of *dsx*. Consequently, homing of the CHE into this sequence impeded the generation of the female specific isoform of Dsx (DsxF), which successfully resulted in complete collapses of caged populations within 7-11 generations (Kyrou et al., 2018). Notably, while sequencing the gRNA target site of mosquitos from several generations showed the emergence of NHEJ-based indels, all mutations resulted in a loss of function of DsxF, owing to the highly conserved and tightly controlled virtue of the target sequence that does not tolerate sequence-variability (Kyrou et al., 2018). However, since such evolutionary conserved sequences are typically involved in essential developmental processes, there is little chance that this strategy can be transferred to replacement homing drives.

In view of the limited applicability of some of the above-mentioned approaches to avoid comprehensively the induced resistance allele formation in homing-based CRISPR/Cas9 gene drives for replacement strategies, the idea to use the CIRSPR/Cas9 technology for the bioengineering of interference-based gene drive systems has gained attention (Oberhofer et al., 2019). As described and explained at length in section 2.3.5, naturally occurring interference-based selfish genetic elements (SGEs) usually lead by default to a replacement situation by typically following a toxin-antidote principle through which they achieve super-Mendelian inheritance without being reliant on the resistance-prone homing mechanism (Burt and Crisanti, 2018; Burt and Trivers, 2008). Inspired by the MEDEA (maternal effect dominant embryonic arrest) system observed in *T. castaneum* (Beeman and Friesen, 1999), a first synthetic toxin-antidote-based SGE was realized in form of an artificial *MEDEA*-locus in *D. melanogaster* (section 2.3.5; Chen et al., 2007). However, although the system has recently been successfully transferred to the fruit crop pest *D. suzukki* (Buchman et al., 2018), its translation to other, more distantly related insect species has found to be difficult due to the drosophilid-specific components used therein.

In this regard, Oberhofer et al. most recently developed a CRISPR/Cas-based, MEDEA-like SGE termed *CleaveR* (*ClvR*) – acronym for Cleave and Rescue – which should be of broader applicability, due to its straightforwardly adaptable constituents and simple molecular makeup (Oberhofer et al., 2019). The *ClvR* is an autosomal two-component driver allele consisting of the toxin-like element "Cleaver", which encodes for a germline-expressed Cas9 and gRNAs designed to target and thereby knockout an essential haplosufficient housekeeping gene located *in trans* on the X chromosome(s), and the tightly linked

antidote-like "Rescue", which contains a recoded variant of the vital gene that is unrecognizable for the gRNAs of the “Cleaver”. As a result, multiplexed targeting and cleavage of the essential gene in the germline leads to NHEJ-based creation of loss-of-function alleles (LOF) of the X-chromosomal essential gene in the gametes. Moreover, using the *nanos* promoter for *cas9* expression leads to maternal carryover of the Cas9-gRNA RNPs into the embryos. Consequently, all wild-type alleles will be transmuted to LOF-alleles, resulting in lethality of all offspring lacking the *ClvR* and, therefore, leading to a progressive replacement of the population and spread of a *ClvR*-associated payload gene. While cleavage resistant in-frame alleles at the target sites that maintain the function of the vital gene can theoretically form, their impact on the drive is assumed to be rather mild as they do not confer a decisive advantage over possessing the recoded rescuing allele of *ClvR*, given that the *ClvR*-associated fitness costs are modest.

4.4 Next generation gene drives: temporal or spatial restriction of the spread

In the previous section, different options were discussed as to how the self-induced genetic instability and associated dysfunction of the first developed “simple” homing-based overreplication gene drive systems can be remedied, or how these deficiencies can be circumvented by changing the strategy to an interference-based gene drive method. However, while improved and comparatively inexpensive, such gene drive systems where only a single seeding release of a few transgenic insects is sufficient to initiate the propagation and fixation of a SGE into a population also have the capacity to spread into interbreeding neighboring populations, or to closely related non-target species to which low levels of gene flow exist, and could, therefore, theoretically disseminate over entire continents or even globally (Noble et al., 2018; Rode et al., 2019). Consequently, the application of such, so-called, unrestricted self-sustaining low threshold gene drives is considered to be unsuitable for the suppression or replacement of a single defined population, as they are not only associated with unforeseeable risks for the ecosystems, but also make region- or country-specific differentiated decisions on their regulation impossible (Committee on Gene Drive Research in Non-Human Organisms: Recommendations for Responsible Conduct et al., 2016). For example, attempts to contain the prevalence of an invasive species in one country could, over time, eventually jeopardize its existence in those countries, in which it is endemic. On this occasion, the development of novel “next generation” gene drive systems, whose spread is either temporarily or spatially restricted, has recently sparked strong interest among scientists and stakeholders, as they would significantly reduce the risk of unintended genetic migration of the SGE by enabling to confine its drive to a given population (Dhole et al., 2018; Esvelt et al., 2014; Marshall and Hay, 2012). Hence, methodically sound self-limiting next generation gene drives might therefore have the potential to

set the stage for the elaboration of national and international regulatory frameworks in due consideration of ethical, social, and environmental aspects. The currently conceptualized and developed next generation self-limiting gene drives can be subcategorized depending on whether the intention of limiting their action spectrum is to achieve a temporal or spatial restriction, and if they require a single release of only a small number (low threshold), or multiple releases with very large quantities (high threshold) of carrier organisms to be able to drive into a population.

With respect to the development of a temporally restricted (or self-exhausting) SGE, Noble et al. recently designed and modelled the behavior of a HCGD for population replacement, which they termed daisy-chain drive (Noble et al., 2019). The name refers to its modular composition, which comprises a set of unlinked – preferably on separate chromosomes located – genetic elements organized and programmed such that each preceding element in the chain drives the overreplication of the subsequent module, with the final element bearing the payload gene to be spread. Following this scenario, the copy number of the last element will rapidly increase close to fixation within the population. However, owing to the system's genetic makeup the first element does not receive any drive itself. Consequently, the release sets a generational clock during which the current lowermost element will gradually segregate from the daisy chain in filial generations and will eventually disappear from the population due to its associated fitness cost. This in turn prevents the overreplication of the next element and so on and so forth, until drive comes to a complete halt and all elements are lost from the population again. In order to avoid increased levels of recombination between the elements, it will be important to engineer such systems with the least possible degree of sequence homology (Noble et al., 2019). Since only a few transgenic individuals are needed to trigger the strong but transient drive, daisy-chain drives, also referred to as multi-layer split homing drives, are to be classified as low threshold drives.

As opposed to this, currently developed gene drives whose spread is spatially restricted are collectively high threshold drives. One possible strategy of how spatial restriction can be achieved has most recently been described in *Aedes aegypti* by Li et al., in which the two essential homing drive components (*cas9* gene and gRNA) are separated into two non-autonomous elements that are inserted into distinct genomic loci (Li et al., 2020). With this in mind, the authors generated a split homing drive system where male mosquitos carry both, an element A in locus 1 expressing *cas9,* and a homing element B in locus 2 encoding for the gRNA and a payload gene. Consecutive large-scale releases of males double-homozygous for both elements would consequently lead to a high frequency of co-occurrence of A and B in individuals of the target population, which enables efficient homing and spread of payload gene containing element B. Importantly, this necessity of periodically releasing huge quantities of the split drive males for the system to work, is at the same time also the underlying reason for its spatial restriction, as A and B will rapidly segregate when accidently introduced into a neighboring wild-type population and will

therefore ultimately vanish by virtue of natural selection due to fitness effects, preventing further spread (Champer et al., 2019; Li et al., 2020).

A similar effect of spatial restriction can also be achieved on the basis of a toxin-antidot-based interference split drive, as previously shown theoretically (Gould et al., 2008), and lately demonstrated empirically in a proof-of-concept study in *D. melanogaster* (Webster et al., 2020). This so-called Killer-Rescue system (K-R) is composed of a killer construct (K) whose lethal effect can be neutralized in the presence of a second unlinked rescue construct (R) placed on another chromosome. For the engineering of K-R, the well-known yeast-derived *Gal4/UAS* repressible binary expression system was employed. To induce death in flies carrying only the K allele, K was designed as an autocidal genetic construct containing a *UAS-Gal4* positive feedback loop, which results in the production of lethal amounts of the Gal4 transcription factor. To rescue the lethal phenotype of K, R comprises a *UAS* construct that controls the expression of the Gal4-inhibitor Gal80, which is initiated via Gal4 in co-presence of K. As is typical for a high threshold drive system, this study has also shown that either a single release at a ratio of 2:1 (homozygous K-R individuals to target population size), or several smaller releases at ratios of 1:2, are necessary to provide a sufficient initial density of K and R alleles in the population to enable K to drive R with its interlinked payload gene over a reasonable number of generations. In analogy to the split homing drive system described above, a low gene flow of K and R alleles into a sister population should not be critical due to rapid Mendelian segregation, and disappearance though natural selection (Gould et al., 2008; Webster et al., 2020).

A further strategy for which the toxin-antidote principle was harnessed to create a regionally limitable interference-based replacement drive is an extension of the MEDEA system described in sections 2.3.5 and 4.3, and is referred to as double-MEDEA (Akbari et al., 2013; Wimmer, 2013). In contrast to a one-fold MEDEA system, the double-MEDEA approach is made up of two trans-interacting toxin-antidote constructs situated on different chromosomes. The first construct comprises a maternally expressed toxin A and encodes for a zygotically produced antidote B, whereas the second construct contains a maternally expressed toxin B and a zygotic antidote A. Since all eggs from double-MEDEA mothers will therefore be dosed with both lethal toxins, only those offspring will survive that also inherit both transgenic constructs, either from their MEDEA-mother, a carrier father, or a combination thereof. Consequently, while all offspring of double-homozygous MEDEA mothers can develop normally, the survival rate of crosses between two double-heterozygous parents, or a double-heterozygous mother and a wild-type father, amounts to only 56% and 25%, respectively (Wimmer, 2013). Hence, as can be deducted from this numbers, for a double-MEDEA system to be able to drive, releases of double-homozygous males at super-threshold frequencies are required, which in turn implies its spatially limited activity (Marshall and Akbari, 2016). This effect, where heterozygotes or their descendants are at a disadvantage compared to

their corresponding homozygotes is generally referred to as underdominance, wherefore approaches such as the double-MEDEA system are also designated as underdominance gene drives (Akbari et al., 2013; Marshall and Akbari, 2016; Wimmer, 2013). However, until now a double-MEDEA-based underdominance system has only been successfully implemented in *D. melanogaster*, since it requires in-depth knowledge of maternally expressed and early zygotic genes, which is often not readily available in non-model organisms (Akbari et al., 2013; Oberhofer et al., 2019; Wimmer, 2013).

Furthermore, the double-MEDEA system can also be converted into a population suppression approach named MEDEA underdominance sex-chromosome associate (MEDUSA), when placing one of the two reciprocal toxin-antidote constructs on the X or an autosome and the other one on the Y chromosome, and exchanging promoter of Y-linked toxin for a promoter that is active in the early embryo (Marshall and Hay, 2014). As a result, only males that inherited the combination of the construct-bearing-Y and the other construct-bearing chromosome, will be able to survive, while all females die, leading to an all-male population collapse.

Another key strength of the high threshold drives described here, in addition to their spatial confinement, is that they can also be reversed by the mass release of wild-type individuals (drive out) (Li et al., 2020; Webster et al., 2020; Wimmer, 2013). However, in the case of the double-MEDEA underdominance replacement drive, this would also require the release of large quantities of females, which is problematic when targeting mosquitoes, as it is the females that suck blood and thereby transmit diseases (Wimmer, 2013).

5 References – for chapters 2, 3.2 (without 3.2.2), and 4

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6 Appendix

Abbreviations

5'T^0	5 prime end thymine at position 0
A	adenine
aa	amino acid
ACP	accessory gland protein
ACP70A	Accessory gland protein 70A
AEL	after egg laying
Asn; N	asparagine
Asp; D	aspartate (aspartic acid)
attB	*bacterial attachment site*
attP	*phage attachment site*
AW-IPM	area wide integrated pest management
bp	base pair
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis subsp. israelensis*
C	cytosine
Cas9	CRISPR-associated DNA nuclease9
Cc	*Ceratitis capitata*
Cctra	*Ceratitis capitata transformer*
cDNA	complementary DNA
CDS	coding sequence
CFP	cyan fluorescent protein
Cre	cyclization recombinase
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Cry4A	Crystal 4A toxin
D	s. Asp
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DDT	dichlorodiphenyl-trichloroethane
dgRNA	double guided RNA
DICE	dual integrase cassette exchange
Dm	*Drosophila melanogaster*
Dm-hsp70	*Dm* regulatory region of *heat-shock protein 70*
DNA	deoxyribonucleic acid
DSB	double-strand break
dsDNA	double-stranded DNA
DsRed	*Discosoma sp*. Red
DUP99B	Ductus ejaculatorius peptide 99B
EGFP	enhanced GFP
FBS	fetal bovine serum
Flp	flippase
fMet	N-Formylmethionine
FRT	*flippase recognition target*
fsRIDL	female-specific RIDL
G	guanine
G	s. Gly
GFP	green fluorescent protein
Gly; G	glycine
GOI	gene of interest
gRNA	guide RNA
GSS	genetic sexing strain
H	s. His
H2Av	histone H2A variant
HDR	homology directed repair
HE	homing endonuclease
HEDG	homing endonuclease gene drive
HEG	homing endonuclease gene
hidAla5	*head involution defective* Alanine 5
His; H	histidine
HITI	homology-independent target integration
HR	homologous recombination
hs43	heat-shock basal promoter of *Dm* regulatory region of *heat-shock protein 70*
I	s. Ile

Ile; I	isoleucine
indel	insertion and deletion
IPM	integrated pest management
IPTG	Isopropyl β-D-1-thiogalactopyranoside
iRMCE	integrase-RMCE
ITR	*inverted terminal repeat*
IVM	integrated vector management
kbp	kilobase pair(s)
kDa	kilodaltons
L1	first larval instar
loxP	*locus of crossing over (x), P1*
Mbp	megabase pair(s)
MNL	Monalysin
mRNA	messenger RNA
MSC	multiple cloning site
MWCO	molecular weight cut-off
N	s. Asn
NHEJ	non-homologous end joining
NHS	normal horse serum
NMWL	nominal molecular weight limit
o.n.	over night
ORF	open reading frame
P	s. Pro
PAM	*protospacer adjacent motif*
pBac	*piggyBac* (transformation vector)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pen/ Strep	Penicillin/ Streptomycin
pgSIT	precision guided SIT
PMR	post mating response
Pol	polymerase
pre-pro-SP	immature Sex-Peptide with N-terminal signal peptide and pro-peptide
Pro; P	proline
pro-Cry4A	inactive Cry4A pro-toxin
pro-MNL	inactive MNL pro-toxin with N-terminal pro-peptide
PUb	*polyubiquitin*
RBE	*recombinase binding element*
rcf	relative centrifugal force
RDF	recombination directionality factor
rDNA	ribosomal DNA
RFP	red fluorescent protein
RIDL	release of insects carrying a Dominant Lethal
RMCE	recombinase-mediated cassette exchange
RNA	ribonucleic acid
RT	room temperature
RTS	*recognition target site*
RVD	repeat variable di-residue
S	s. Ser
S2 cells	Schneider 2 cells
Ser; S	serine
SGE	selfish genetic element
SIT	Sterile Insect Technique
SP	Sex-Peptide
SP-PRS	Sex-Peptide-derived protease recognition site
SSB	single-strand break
ssDNA	singe-stranded DNA
SSR	site-specific recombinase
SV40PolyA	*stop/poly adenylation-signal* (Herpes simplex virus)
Sxl	*Sex lethal*
T	thymine
T	s. Tyr
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nulcease
Tc	*Tribolium castaneum*
Tc-hsp68	*Tc* regulatory region of *heat-shock protein 68*
TE	transposable element

tracrRNA	trans-acting CRISPR RNA
TRE	tTA responsive element
TREp	tTA responsive element + *p-Element* basal promoter
TSS	transgenic sexing strain
tTA	tetracycline-controlled transactivator
Tyr; T	tyrosine
U	uracil
UAS	*upstream activation sequence*
UTR	untranslated region
wt	wild-type
YFP	yellow fluorescent protein
ZFN	zinc-finger nuclease
β2t, β2tub	β2-tubulin
βTub	βTubulin 85D
ΦC31-Int	integrase phiC31
pro-MNL^{SP-PRS}	enginnered pro-MNL
CHE	CRISPR/Cas9 homing element
HCGD	homing CRISPR/Cas9 gene drive

Curriculum vitae

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Work & Research Experiences

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Research

- Conceptualization, design, and execution of research projects
- Data collection, (statistical) analysis and evaluation
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Teaching

- Development and implementation of practical courses and tutorials
  - *"Entwicklungsbiologie der Tiere"*
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- Conceptualization and supervision of master, bachelor, and placement student research projects; Revision and evaluation of theses and reports
- Initial training of doctoral candidates and supervision of collaboration partners in Germany and abroad

Management

- Organization, administration and logistics of laboratory responsibilities
- Setup and maintenance of laboratory infrastructure
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Laboratory internships (master student)

03 – 04/2012 Department of Developmental Biology, Max-Planck-Institute for Biophysical Chemistry
Supervised by Prof. Dr. Michael Kessel
"Mad2l2-deficient mouse embryonic stem cells deviate in vitro to primitive endoderm"

01 – 02/2012 Department of Developmental Biochemistry, University Medical Center Göttingen
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"Germ plasm formation in zebrafish"

International Collaborations

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- Co-ordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (FAO/IAEA Insect Pest Control Section) on *"Comparing Rearing Efficiency and Competitiveness of Sterile Male Strains Produced by Genetic, Transgenic or Symbiont-Based Technologies"*

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Memberships, Scholarships & Awards

Memberships	• German Society of Developmental Biology • Insect Genetic Technologies Research Coordination Network (IGTRCN)
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Publications & Selected Presentations

Publications

Farnworth M.S., **Eckermann K.N.**, Bucher G. (2020). Sequence heterochrony led to a gain of functionality in an immature stage of the central complex: A fly–beetle insight. *Plos Biol.* 18(10):e3000881.

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Book Chapter

Farnworth, M.S., **Eckermann, K.N.**, Ahmed, H.M.M., Mühlen, D.S., He, B., Bucher, G. (2020). The red flour beetle as model for comparative neural development: Genome editing to mark neural cells in *Tribolium* brain development, in: Sprecher, S. (Ed.), Brain Development: Methods and Protocols, Second Edition. Springer New York, New York, USA.

Oral Presentation

Eckermann, K.N., Ahmed, H.M.M., KaramiNejadRanjbar, M., Isah, M.D., Curril, I.M., Wimmer, E.A. (2016). A novel endonuclease-based transgenic male reproductive sterility system causing chromosome shredding to improve the Sterile Insect Technique. 3rd International Symposium of Tephritid Workers of Europe, Africa and the Middle East (TEAM), 2016, in Stellenbosch, South Africa.

Poster presentation

Eckermann, K.N., KaramiNejadRanjbar, M., Dippel, S., Ogaugwu, C.E., Wimmer, E.A. (2014). Hyperactive *piggyBac* transposase shows improved germ-line transformation rate in agricultural insect pests. 9th International Symposium on Fruit Flies of Economic Importance (ISFFEI), 2014, in Bangkok, Thailand.

Extracurricular Activities and Outreach

Stand coordination: Presentation and demonstration of biotechnological innovations in the Sterile Insect Technique. BIOTECHNICA fair, 2013, in Hannover, Germany.

Organizing member of the Göttingen Center for Molecular Biosciences (GZMB) Summer Symposium (2014)

Participation in the International Symposium on Arthropod-borne infectious diseases and Arthropods as disease agents in human and animal health, in Berlin, Germany (2016).

References

Available upon request.