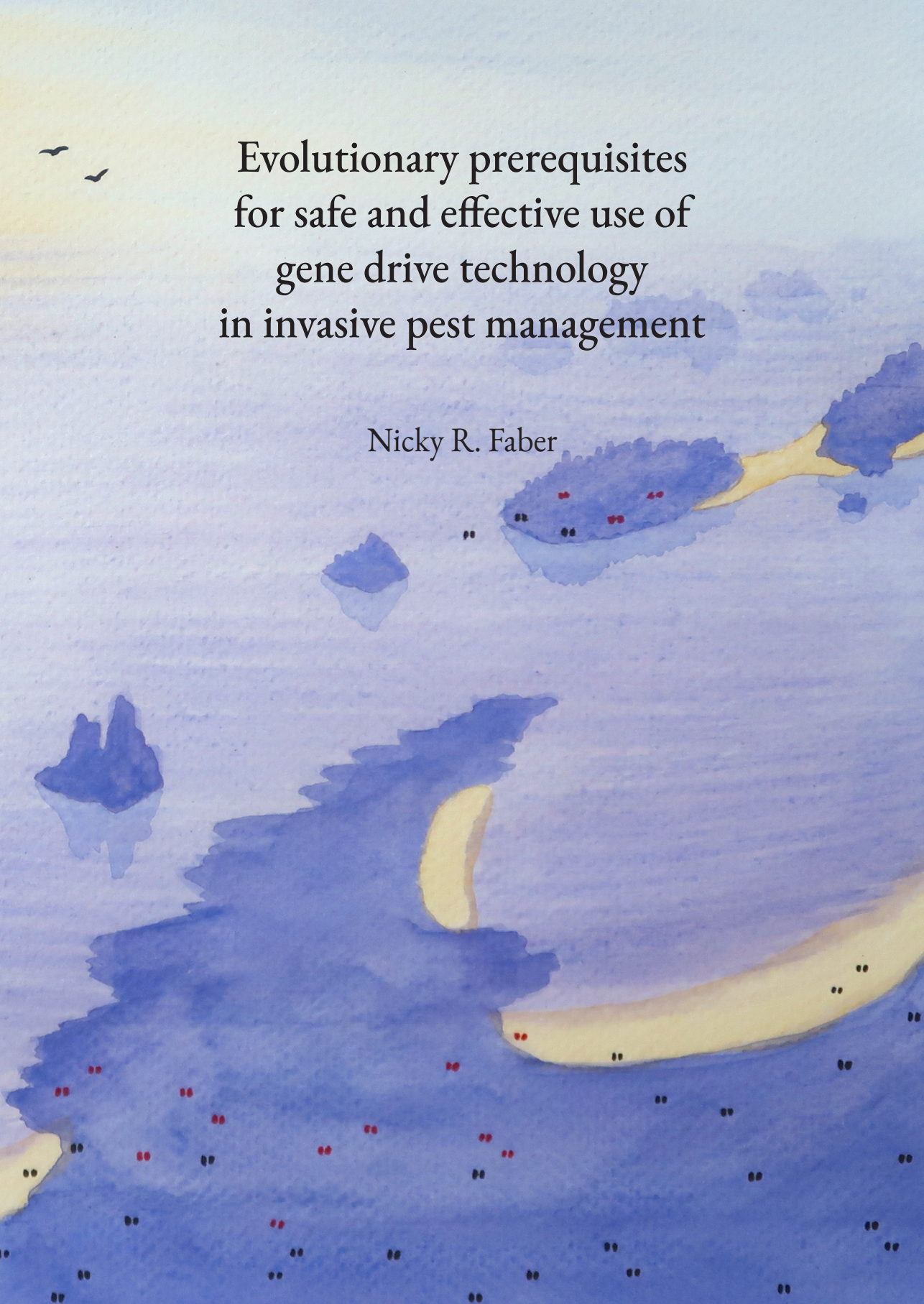
Evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management

Nicky R. Faber



Propositions

1. Gene drives are not scary (yet) (this thesis).
2. Genetic biocontrol leads to a genetic form of "space junk" (this thesis).

3. A hard PhD deadline prevents neglect and power abuse.
4. Conducting experiments requires more mental resilience than hiking 2650 miles.
5. PhD candidates and postdocs need contractual rights to unpaid leave.

6. Banning advertising promotes sustainability.
7. To combat the accumulation of toilet paper in nature, one must build toilets.

Propositions belonging to the thesis, entitled

Evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management

Nicky R. Faber
Wageningen, 25 September 2026

Evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management

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Evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management

Nicky R. Faber

Thesis

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"I must hold in balance the sense of the futility of effort and the sense of the necessity to struggle; the conviction of the inevitability of failure and still the determination to "succeed" – and, more than these, the contradiction between the dead hand of the past and the high intentions of the future."

— F. Scott Fitzgerald, The Crack-up

"The dodo itself stands as the best emblem of this general truth–that insular evolution often involves transforming an adventurous, high-flying ancestor species into a grounded descendant, no longer capable of going anywhere but extinct. It's our reminder that insular evolution, for all its wondrousness, tends to be a one-way tunnel toward doom."

— David Quammen, The Song of the Dodo

Chapter 1

General introduction

This chapter is partly based on:

Faber, N. R., Ashok, K., Venkatesan, T., Wertheim, B., & Bulgarella, M. (2025). Leveraging advances in RNAi and CRISPR for improved biological pest control. *Current Opinion in Insect Science*, 101453. https://doi.org/https://doi.org/10.1016/j.cois.2025.101453

I

1.1 Invasive species

The dodo is probably the most charismatic example of a species that has gone extinct due to human activity. Many people believe that the dodo was hunted to extinction because they were excessively stupid birds; in fact, this belief probably contributed significantly to the dodo's fame (Turvey & Cheke, 2008). The following quote from Smith (1819), an old children's natural history guidebook, illustrates this ungenerous sentiment:

> *The Dodo,* Didus*, is a bird that inhabits some of the islands of the East Indies. Its history is little known; but if the representation of it be at all just, this is the ugliest and most disgusting of birds, resembling in its appearance one of those bloated and unwieldy persons who by a long course of vicious and gross indulgences are become a libel on the human figure.*

Contrary to popular belief, however, there are three major causes behind the extinction of the dodo: excessive hunting and habitat destruction by humans, and what is perhaps surprisingly thought to be the most important factor: predation by invasive species (Cheke, 1987). European colonists unintentionally introduced rats and monkeys to Mauritius, the former as unwelcome stowaways on ships, the latter likely as rejected pets. Furthermore, they intentionally introduced pigs, goats, deer, and cattle to serve as food for future sailors (Cheke & Parish, 2020). Predation on the dodo's eggs and nestlings by invasive rats and pigs is most likely the primary force that ultimately drove the dodo to extinction (Cheke, 1987).



Figure 1.1. A portrait of a dodo painted by Cornelis Saftleven in 1638. This is potentially one of the last ever depictions based on a living dodo (Hume, 2006).

Invasive species are still a major and increasing problem today (Díaz & Malhi, 2022; Mooney et al., 2005; Pejchar & Mooney, 2009; Sala et al., 2000; Schaffner et al., 2013; Seebens et al., 2020). The problems caused by invasive species include the threatening of native biodiversity through pre-

dation or resource competition, spreading disease to humans or native species, and damaging crops or harming livestock. Well-known examples of such invasive species include rats and mice on many islands worldwide, rabbits, cane toads, and cats in Australia, stoats and possums in New Zealand, and many mosquitoes, fruit flies, and other insect species worldwide (Luque et al., 2014).

In many cases, invasive species are so prolific and widely distributed that conventional management techniques fail to address the scope of the problem. Moreover, current control methods often cause harmful side effects; for example, insecticides and rodenticides often affect non-target species including humans. Additionally, conventional control methods raise ethical concerns due to reduced animal welfare; for example, shooting, poisoning, trapping, or using viral agents often cause considerable animal suffering. For invasive insects, classical biocontrol by releasing natural enemies has proven to be an efficient method of control, enabling the replacement of chemical pesticides entirely in some crop systems (Mason, 2021; van Lenteren et al., 2018). Despite the successes, biological control does not always provide effective pest management, and developing these approaches is a time-consuming and labour-intensive process. Overall, to achieve sustainable invasive pest management in some cases, we need more effective, species-specific, and ethical methods of control. In recent years, novel molecular technologies have provided opportunities to achieve this.

In this thesis, I investigate a technology termed "genetic biocontrol" that I believe to be a promising candidate, because it potentially meets all three of these criteria. Although it is too late to save the dodo from extinction, a similar example of a threatened species that perhaps equals the dodo in terms of charisma (or, goofiness), and which has no chance at survival while invasive species persist is the kākāpō: a large, flightless parrot in New Zealand that only survives on small off-shore islands, where rat and mouse eradication programmes were able to be successful. Perhaps with genetic biocontrol of invasive species, the kākāpō and many other threatened species could reclaim significant parts of their former range and not need such intensive management as they do now.

1.2 Genetic biocontrol

Genetic biocontrol consists of a range of different pest management strategies, all of which employ a genetic mechanism to reduce pest populations in size or reduce their harm directly. For population control, these strategies usually include sterilising adult individuals, inducing lethal genotypes in offspring to arrest their development, or biasing the sex ratio of a population towards males. With all of these methods, less offspring is produced in the next generation and the population is reduced in size.

The idea of using genetics for the purpose of biocontrol has been around since the 1940s (Serebrovsky, 1969, translated from Russian to English after its original publication in 1940). The first practical application of the mechanism was in the 1950s, when a strategy called sterile insect technique was first tested for the screw-worm fly (*Cochliomyia hominivorax*), a flesh-eating parasite of warm-blooded animals including livestock (Klassen et al., 2021). Sterile insect technique reduces pest population size through the mass releases of sterilised males. These males are irradiated, causing dominant lethal mutations in reproductive cells, among others, thus effectively rendering them sterile. This strategy has successfully eliminated the screw-worm fly across much of North and Central America (which are part of its native range), and is also applied for the control of many other insect species (Craig et al., 1960; Klassen et al., 2021). However, to have a measurable impact on population size, usually, millions of sterile males must be released on a weekly basis to rid a relatively small area such as a neighbourhood of a pest. This technology is therefore extremely labour-intensive and expensive. Additionally, both the irradiation and mass-rearing of insects often leads to unwanted fitness costs, resulting in a reduced ability to compete with wild males (Lance & McInnis, 2021). Unfortunately, due to the constant human-mediated movement of livestock, occasional resurgences occur and the process begins anew (Zaldivar-Gomez et al., 2025).

More powerful and precise genetic biocontrol strategies using genome editing have been under development in recent decades, but were often hard to engineer or not efficient enough for practical applications until the advent of CRISPR-based genome editing in 2012 (Jinek et al., 2012; Simoni et al., 2014). Since then, a myriad of genetic biocontrol strategies has been developed, either to control pest populations directly or to precisely modify them to remove undesirable traits such as the ability to transmit disease (Hay et al.,

2021).

Genetic biocontrol strategies span a spectrum primarily defined by a trade-off between controllability and power. On the most controllable end are strategies that require continuous mass releases of modified insects carrying deleterious traits, such as a CRISPR-based versions of sterile insect technique (Figure 1.2A) (Alphey & Andreasen, 2002; Kandul et al., 2019). These systems provide benefits over conventional sterile insect technique because they avoid fitness costs from off-target effects of radiation and can aid in producing male insects through sex-biasing genetics. However, rearing the large amount of insects necessary for repeated releases is still a major limiting factor on the power of these technologies (Bouyer & Vreysen, 2019).

On the most powerful end of the genetic biocontrol spectrum lie CRISPR-based homing gene drives, which are selfish genetic elements capable of spreading through a population themselves, theoretically even from a single, small release of individuals (Esvelt et al., 2014). In general, gene drives can achieve this spread by biasing inheritance in their own favour over multiple generations in a sexually reproducing population (Figure 1.2D). Concurrently, they can be designed to induce recessive female sterility, thus reducing a pest population in size (Kyrou et al., 2018; E. Yang et al., 2022). For most species, females are the limiting factor on the reproductive potential of the population, so targeting them in genetic biocontrol is more effective than inducing sterility in males. Although this technology is drastic and comes with obvious containment and ethical challenges, it is under consideration for a few pests with large, global impacts, such as malaria-transmitting mosquitoes (*Anopheles* spp.) and the screw-worm fly (Kaebnick et al., 2025).

Interestingly, to leverage the strengths of both sides of the spectrum, many hybrid systems combining features of genetic SIT and gene drives have been proposed to impose spatially or temporally limited population control (Han & Champer, 2025). For example, sterile insect technique–like strategies can be made more powerful if they only eliminate female offspring, thus allowing the genetic modification to linger in male offspring for several generations, leading to somewhat more sustained population control (Figure 1.2B) (Alphey et al., 2008; Fu et al., 2007). Again, the periodic addition of more modified individuals is necessary to reduce the pest population in size (though less than would be necessary for standard sterile insect technique), as these systems cannot persist in a population.

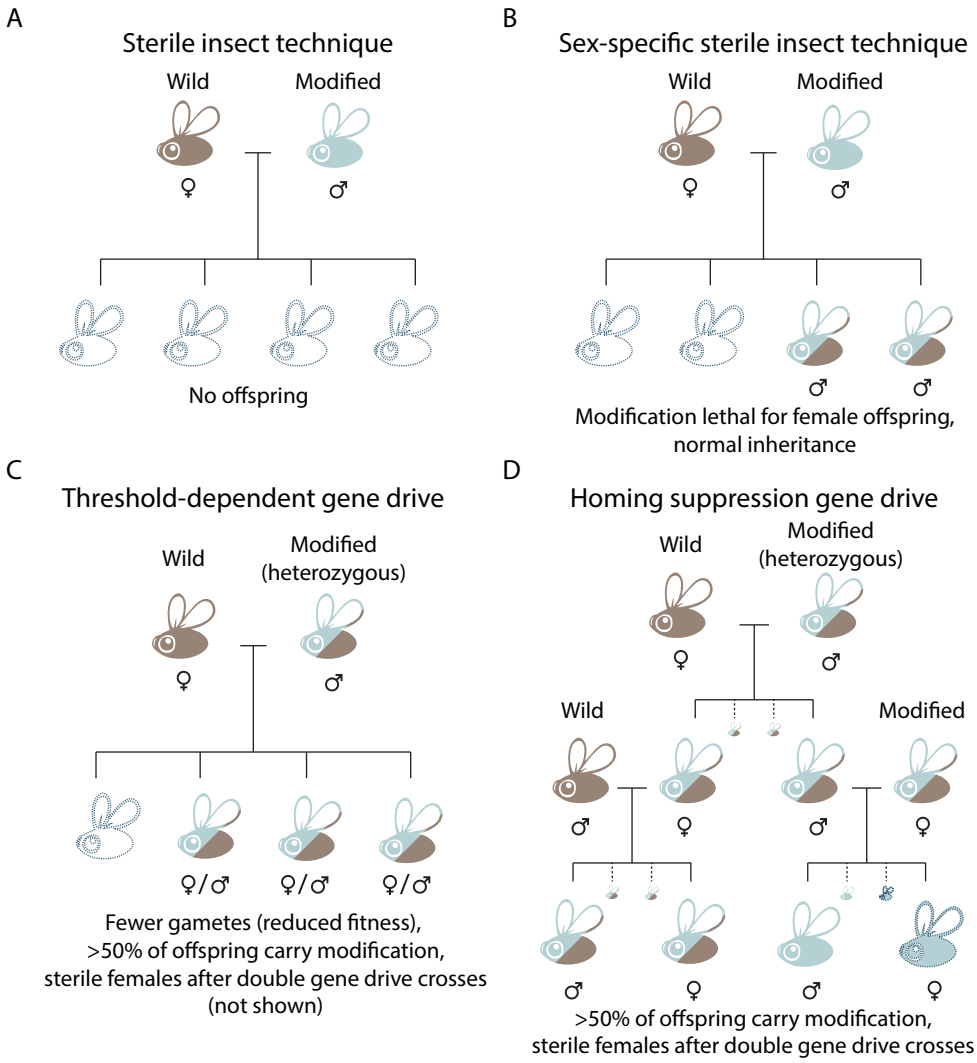


Figure 1.2. Graphical overview of genetic biocontrol strategies. A and B, non–gene drive strategies, C and D, gene drive strategies. A) Genetic versions of sterile insect technique, including precision-guided Sterile Insect Technique (pgSIT) and versions of Release of Insect carrying Dominant Lethal alleles (RIDL). B) Female-specific sterile insect technique strategies, including female-specific Release of Insects carrying Dominant Lethal alleles (fsRIDL). C) Threshold-dependent, or local, gene drive strategies, including toxin-antidote gene drives. D) Homing gene drives, also called global gene drives. Two out of four offspring are pictured smaller, but there is no reduction in fertility in this system.

Other hybrid strategies exist that, besides reducing offspring numbers, bias inheritance in their own favour through gene drive, but in a manner that allows them to increase in frequency only under specific conditions (Figure 1.2C). These gene drives are called threshold-dependent, self-limiting, or local gene drives (as opposed to global homing gene drives) (J. Champer, Champer, et al., 2021; J. Champer, Kim, et al., 2020; Chen et al., 2024; Oberhofer et al., 2019). By design, drive carriers have reduced reproductive potential, so the gene drive can only increase in frequency in a population when above a certain frequency threshold. This height of this threshold depends on the strength of the fitness cost associated with the gene drive (Deredec et al., 2008). When under this threshold, the gene drive will only have a transient impact on the population size but will ultimately be lost. Threshold-dependent gene drives are thought to be safer alternatives to global gene drives, as population control can be achieved in a target population through a large introduction of modified individuals, while occasional escapes to non-target populations are rare enough that the gene drive remains below the critical threshold, and thus be unable to spread.

Which genetic biocontrol strategy might be most suitable in a certain scenario will ultimately depend on the ecological characteristics of the invasive species (Dhole et al., 2020). SIT-like strategies are most suitable for insects that generally mate only once and are easily mass-reared and released (Dyck et al., 2021). Homing gene drives are likely more suitable for long-term control over large, inaccessible areas such as the eradication of rats in New Zealand, cane toads in Australia, or mosquitoes over vast areas (North et al., 2020). Importantly, powerful gene drives are likely desirable for invasive species for which releasing large amounts of individuals will have additional negative ecological impacts. For example, releasing large amounts of sterile male mosquitoes poses no threat to humans as they do not bite animals and do not spread disease, while releasing large amounts of rats on an island will negatively impact native biodiversity because of the added predation pressure.

In this thesis, I focus on homing gene drives as their power is likely required to control populations of invasive species over inaccessible areas in many cases. Although homing gene drives can be classified as global based on their ability to spread through a population from a low frequency, their design is versatile; constructs are highly modular, and spatial or temporal limitations can readily be included in these constructs (Dhole et al., 2019; Min et al., 2017a, 2017b; Noble et al., 2019).

1.3 Homing gene drives

Gene drive is a natural phenomenon whereby a genetic element skews the process of inheritance in its own favour (Werren, 2011). There is a wide range of mechanisms through which natural gene drive can occur; these include extra chromosomes, meiotic segregation distorters, gamete killers, transposable elements, and endosymbionts. Indeed, the principles of these natural gene drives have inspired some synthetic gene drives (J. Champer et al., 2016). The advent of CRISPR-based genome editing has rapidly catalysed gene drive research into a fast-growing field (Esvelt et al., 2014; Gantz & Bier, 2015), although synthetic gene drives were already under development prior to the emergence of CRISPR (Chan et al., 2011).

As mentioned above, homing gene drives essentially perform two functions once released in a population. The first function is to increase in frequency by biasing inheritance in their own favour. To this end, wild-type alleles are converted to gene drive alleles in individuals heterozygous for the gene drive. This process is usually designed to only occur in the germline, so that inheritance is biased without inducing a fitness cost in the soma as well (Figure 1.3A). Two essential gene drive components—Cas9 protein and guide-RNAs (gRNAs) targeting the homologous chromosome at the gene drive insertion locus—are expressed during meiosis and cause a double-stranded break in the wild-type allele. This break is followed by DNA repair. Critically, during meiosis, the key DNA repair pathway is homology-directed repair (or, homologous recombination), meaning that the intact chromosome is used as a template for repair. Effectively, the gene drive is thus copy-pasted onto the homologous chromosome, turning the individual into a homozygote for the gene drive in the germline. This process is called "homing" or "drive conversion".

The second function of a homing gene drive, enabled by the first, is to effect the purpose it was designed for, which can be classified into two broad goals: population modification or population suppression (Esvelt et al., 2014). Population modification can be desirable, for example to immunise a vector against a pathogen that it transmits (such as immunising malaria-transmitting mosquitoes against the malaria parasite) (G.-H. Wang et al., 2024). Similarly, an endangered species could be immunised against a disease that threatens their existence, such as amphibians or chestnut trees that are unable to survive fungal infections (Powell et al., 2019; Rosenblum et al., 2010). In these cases, an additional cargo is added to

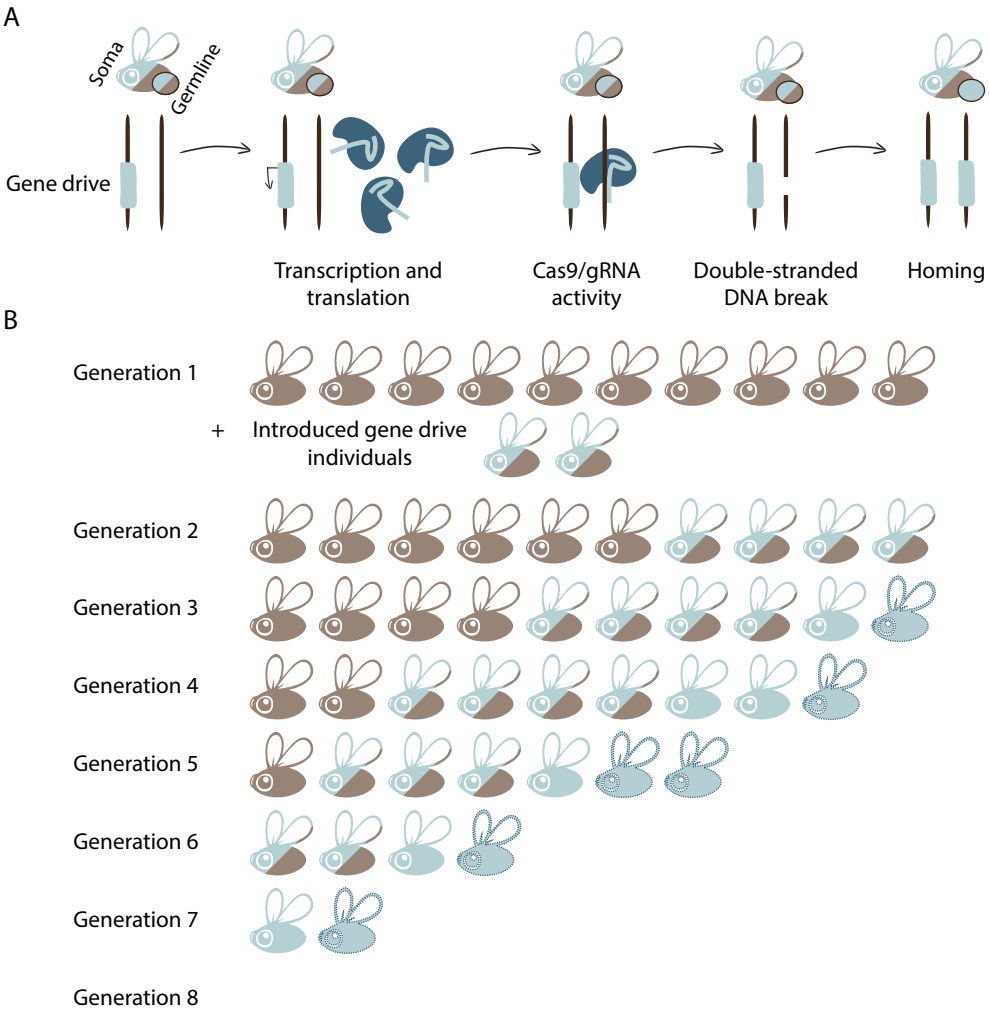


Figure 1.3. Graphical overview of a homing gene drive designed for population suppression. A) The process of homing in gene drive heterozygotes in the germline (initially carrying one gene drive copy and one wild-type copy). B) A homing gene drive spreading over generations in a population, eventually leading to population elimination. A dotted dark blue outline indicates sterile females.

the construct of the gene drive, which can be a single or multiple genes that confer resistance to the disease. In the case of population suppression, which intends the removal of

(part of) a population from its invasive range, the gene drive is designed to induce a significant, usually recessive and sex-specific, fitness cost to its bearer (Esvelt et al., 2014). By only causing such a specific fitness cost, a gene drive can remain hidden from selection for as long as possible while it spreads. The fitness cost is usually achieved by inserting the gene drive into a haplosufficient female fertility gene (meaning one functioning copy of this gene is enough for full fertility), thus disrupting the function of one copy. Once the gene drive has reached a significant frequency, gene drive homozygous females will start appearing. As they are sterile, the pest population will be reduced in size, potentially to the point of population eradication (Figure 1.3B).

After an initial demonstration in fruit flies in 2015 (Gantz & Bier, 2015), homing gene drives were quickly built in yeast (DiCarlo et al., 2015), two mosquito species (Gantz et al., 2015; Hammond et al., 2016), and mice (Grunwald et al., 2019). For all species except mice, gene drive inheritance was significantly higher than 50%, and even over 99% in mosquitoes (DiCarlo et al., 2015; Gantz & Bier, 2015; Gantz et al., 2015; Hammond et al., 2016), meaning that 99% of the offspring of a heterozygous gene drive carrier inherits the gene drive. Recent cage trials in mosquitoes have shown that a gene drive can become fixed in the population after around 10 generations and can lead to complete population suppression (Kyrou et al., 2018; Simoni et al., 2020). Nowadays, with a myriad of different constructs having been built in many different species, researchers are starting to look towards field trials for further testing of this technology (Long et al., 2020). However, significant obstacles remain for homing gene drives, as issues have been identified with homing efficiency, evolutionary stability, and biosafety.

1.3.1 Homing efficiency

The first major challenge for homing gene drives is efficiency, as homing gene drives have only been highly efficient in very few species (Verkuijl, 2023). In *Anopheles* mosquitoes, the genus that transmits malaria disease, extremely high drive efficiencies have been achieved (Kyrou et al., 2018), but these same designs have shown reduced efficiency in *Aedes* mosquitoes (M. Li et al., 2020) and *Drosophila melanogaster* (Oberhofer et al., 2018; E. Yang et al., 2022), and low efficiency in the diamondback moth (*Plutella xylostella*) and mice (Pfitzner et al., 2020; X. Xu et al., 2022). Low homing rates can lead to only partial suppression of the target population or, in the worst case, to no suppression at all (Dere-

dec et al., 2008). Gene drive efficiency depends on many design factors: they should be active specifically during meiosis in the germline (when homology-directed repair is the main DNA repair pathway), while avoiding expression at other times or in different tissues (Ang et al., 2025; Verkuijl et al., 2022). Also, the level of Cas9 and gRNA activity is critical, as too little expression will simply lead to fewer double-stranded breaks and thus, fewer opportunities for homing to occur (Weitzel et al., 2021). Fortunately, optimisation of the gene drive construct, for instance by careful selection of the Cas9 promoter, can lead to significant efficiency improvements (M. A. E. Anderson et al., 2023; Du et al., 2024).

Besides difficulties in optimising the gene drive construct, however, inserting a gene drive into the genome of a desired organism is already a considerable challenge. Transformation efficiencies vary among species and require extensive optimisation in many cases, as seen in the parasitoid wasp *Nasonia vitripennis*, a model hymenopteran that has been recalcitrant to transformation in the past (Guerra et al., 2025). Furthermore, many invasive species are difficult to rear in laboratories, such as the honey bee parasite *Varroa destructor* or social insects. The development of viable suppression strategies also requires detailed genetic knowledge for identifying gene targets and construct design, knowledge that is not readily available for many invasive species (Verkuijl et al., 2022). Increasingly, it seems that many instances of "the wheel" on which gene drives depend may need to be reinvented for every species in which a CRISPR-based genetic biocontrol technology is developed (Ang et al., 2025).

Finally, the life history of an organism has critical consequences for the applicability of gene drive–mediated population suppression. An example includes near-exclusive inbreeding in *Varroa destructor*, leading to sterile gene drive homozygotes before the drive has a chance to spread (Faber, Meiborg, et al., 2021). Some species, such as cats, may have generation times or lifespans that are so long that a gene drive cannot provide a meaningful impact to benefit conservation in the near future, which may make its use impractical in the short-term (Birand et al., 2022). Some invasive species, including the Asian hornet (probably among many other insects), are simply so prolific that they could exploit any small inefficiency in a gene drive and thus remain at regular population density (Meiborg et al., 2023). For any species, factors such as spatial population structure, density dependence, mate choice, and polyandry can interfere with the spread and success of homing gene drives (J. Champer, Kim, et al., 2021; Dhole et al., 2020).

In most invasive species, CRISPR-based genome editing has not been tested as of yet, let alone gene drive efficiency. However, as the number of species in which homing gene drives have been tested increases, perhaps more general design principles and optimisation strategies will become apparent (Verkuijl, 2023). Once an efficient gene drive is developed for a given species, the second challenge emerges: ensuring its evolutionary stability.

1.3.2 Evolutionary stability

A second major challenge for homing gene drives is their persistent efficacy in a population. Namely, the homing capability of a gene drive can be undermined by the evolution of so-called resistance alleles (J. Champer et al., 2018; Price et al., 2020). As CRISPR/Cas-based gene drives use a guide-RNA that matches a 20 base pair long sequence on the homologous chromosome, mutations in this target sequence will render de gene drive ineffective. Unfortunately, the mechanism through which resistance alleles are formed is facilitated by the gene drive itself: a double stranded break made by Cas9 is sometimes repaired through end-joining repair instead of the desired homology-directed repair. Here, instead of using the homologous chromosome as repair template, the two loose DNA strands are simply pasted together. This repair pathway is error-prone and is likely to lead to small insertions or deletions (Figure 1.4A). These mutations usually lead to so-called non-functional resistance alleles, that is, alleles that can no longer be targeted by the gene drive but that do not rescue the function of the gene. However, when end-joining repair leads to functional repair of the wild-type gene, but with a different sequence, a functional resistance allele is formed. These resistance alleles have a major selective advantage over the gene drive, and can therefore rescue the population from suppression (Figure 1.4B) (J. Champer et al., 2016). Thus, efficiency and evolutionary stability are inherently linked: the more efficient a gene drive is, the more stable it will likely be.

Without mitigation strategies, resistance alleles initially evolved against all homing gene drives in every organism in which gene drives were tested (DiCarlo et al., 2015; Gantz & Bier, 2015; Gantz et al., 2015; Hammond et al., 2016). The formation of resistance alleles likely happens at two stages: in the germline and in the early embryo (J. Champer et al., 2017). In the germline, the cause is a discrepancy between the window of Cas9 expression and the window during which homology directed repair is the predominant DNA repair pathway, although it remains unclear how wide or narrow this window is. In the early

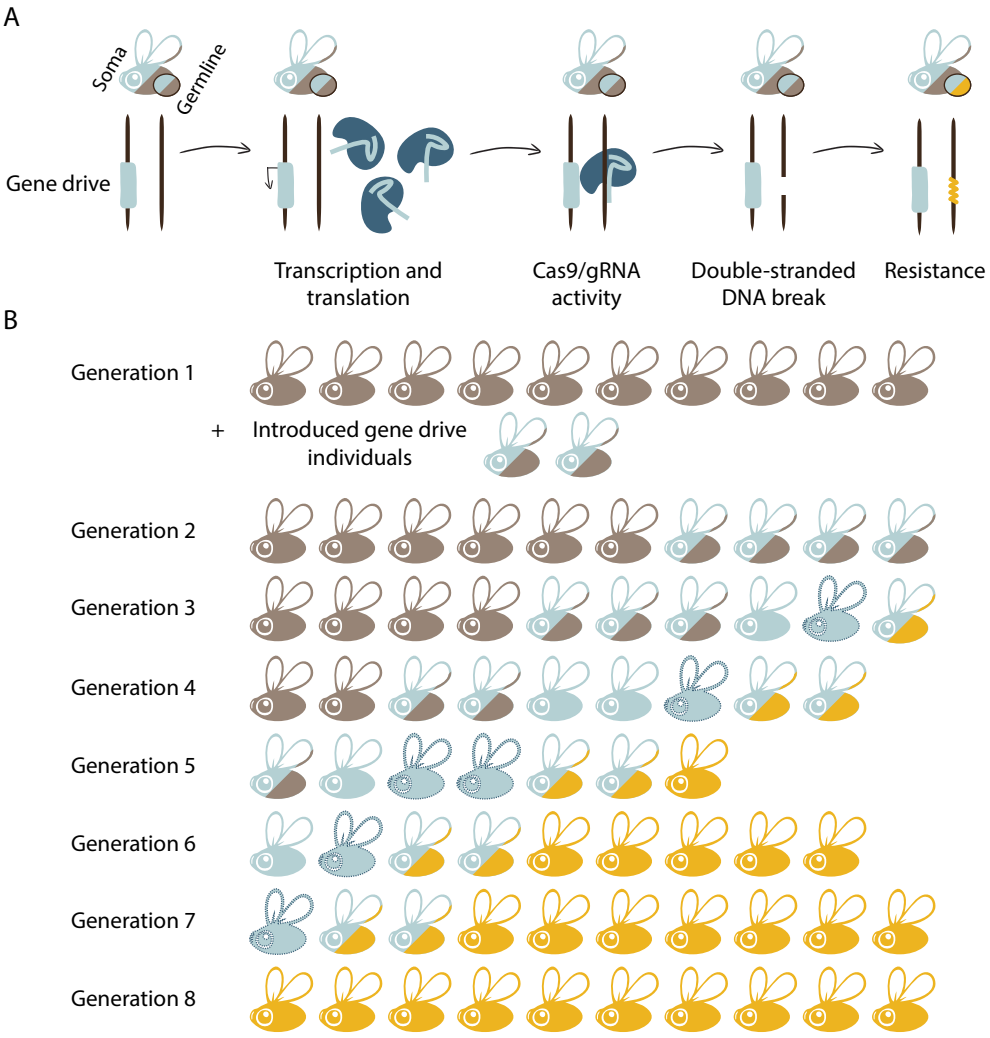


Figure 1.4. Graphical overview of the evolution of resistance against a homing gene drive. A) The process of end-joining and resistance allele formation in gene drive heterozygotes in the germline (initially carrying one gene drive copy and one wild-type copy). B) A homing gene drive spreading over generations in a population, followed by the spread of resistance alleles which rescue the population from suppression. A dotted dark blue outline indicates sterile females.

embryo, cleavage of a wildtype paternal allele can happen because of maternally deposited Cas9. At this time, end-joining usually repairs DNA damage, and thus, a resistance allele

is created that will preclude homing later in the germline.

Several strategies have since been proposed to prevent or remove resistance alleles: finding more specific germline promoters (Pfitzner et al., 2020), targeting extremely conserved sequences so that any resistance allele will likely have a deleterious effect (J. Champer, Yang, et al., 2020; Kyrou et al., 2018), using multiple gRNAs (S. E. Champer et al., 2020; E. Yang et al., 2022), and the use of a cleave-and-rescue mechanisms in addition to or instead of the homing mechanism (J. Champer, Kim, et al., 2020; Dhole et al., 2019; Faber, McFarlane, et al., 2021; Kandul et al., 2021; Oberhofer et al., 2019). Seemingly, with careful engineering, many gene drives can largely prevent the formation of resistance alleles and remain reasonably evolutionarily stable over generations in the laboratory. Of course, the boundaries of these strategies remain to be tested in long-term tests in large populations. With an efficient gene drive that can avoid the formation of resistance, the third and final major challenge for homing gene drives is their biosafety.

1.3.3 Biosafety

As a homing gene drive could theoretically spread indefinitely and invade non-target populations, their design must include a self-limiting factor to guarantee global biosafety (Esvelt & Gemmell, 2017; Esvelt et al., 2014). Physical barriers will almost inevitably be insufficient to prevent natural or illegal human-mediated migration, as demonstrated by the illegal importation of rabbit haemorrhagic virus from Australia to New Zealand by farmers (Boersma et al., 2024; O'Hara, 2006). Therefore, a range of homing gene drives have been designed and, in some cases, tested that are less invasive by nature or mitigate risks to an extent while still retaining more power than previously discussed genetic biocontrol strategies. These designs include constructs that stop spreading after a certain number of generations and are thus self-limiting (Min et al., 2017a, 2017b; Noble et al., 2019), drives that depend on the previous spread of a threshold-dependent gene drive (Dhole et al., 2019; Metzloff et al., 2022), constructs that can halt or remove a gene drive that is already present in a population (B. Wu et al., 2016; X.-R. S. Xu et al., 2020), and constructs that target locally fixed alleles in the invasive population so that the gene drive will not spread in non-target populations (Oh et al., 2021). However, several of these safety mechanisms remain theoretical and have only been demonstrated to work in theoretical population genetic models. Cage and field trials are needed to indicate the best trade-off between the

power and safety of these self-limiting gene drives and will likely also depend on the species and its life history (Wilkins et al., 2018). It is also necessary to test the reliability of these safety designs, as too much variance in performance could still yield unpredictable or undesirable results. Overall, however, it seems that for many species there are still too many inefficiencies in the technology that must be resolved before more extensive effort can be put towards robust and reliable safety mechanisms.

1.4 Open questions in gene drive technology

For certain species, gene drive technology may soon progress to the next phase of evaluation, namely, field trials (Long et al., 2020). However, I contend that several critical questions in gene drive technology remain unresolved and should be addressed before field trials are undertaken. Currently, most studies test gene drives in small cage populations using genetically homogeneous lab strains. These scenarios necessarily simplify the complexity of real-world conditions. Indeed, much is unknown about the efficiency of gene drives in wild, genetically diverse populations, as well as their evolutionary stability over long periods of time, the influence of real-world environmental conditions, or the practical applicability of safety mechanisms such as gene drives targeting locally fixed alleles. Given these knowledge gaps, it is not yet possible to form realistic expectations about the outcomes of field trials. These uncertainties could lead to initial inefficiencies, failures, or unsafe situations, which in turn might have a prematurely negative impact on the public perception and further development of this technology. For example, classical biocontrol of invasive species through introducing natural enemies has led to initial failures and controversies before proper risk-assessment strategies were in place, which has continued to unnecessarily harm public perception of this method to this day, despite its proven increased safety (Barratt et al., 2018). Therefore, it is essential to proceed cautiously in determining whether, and under what conditions, gene drive technology can be used for invasive species control.

1.5 Thesis overview

In this thesis, I investigate the evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management. These evolutionary aspects range from gene

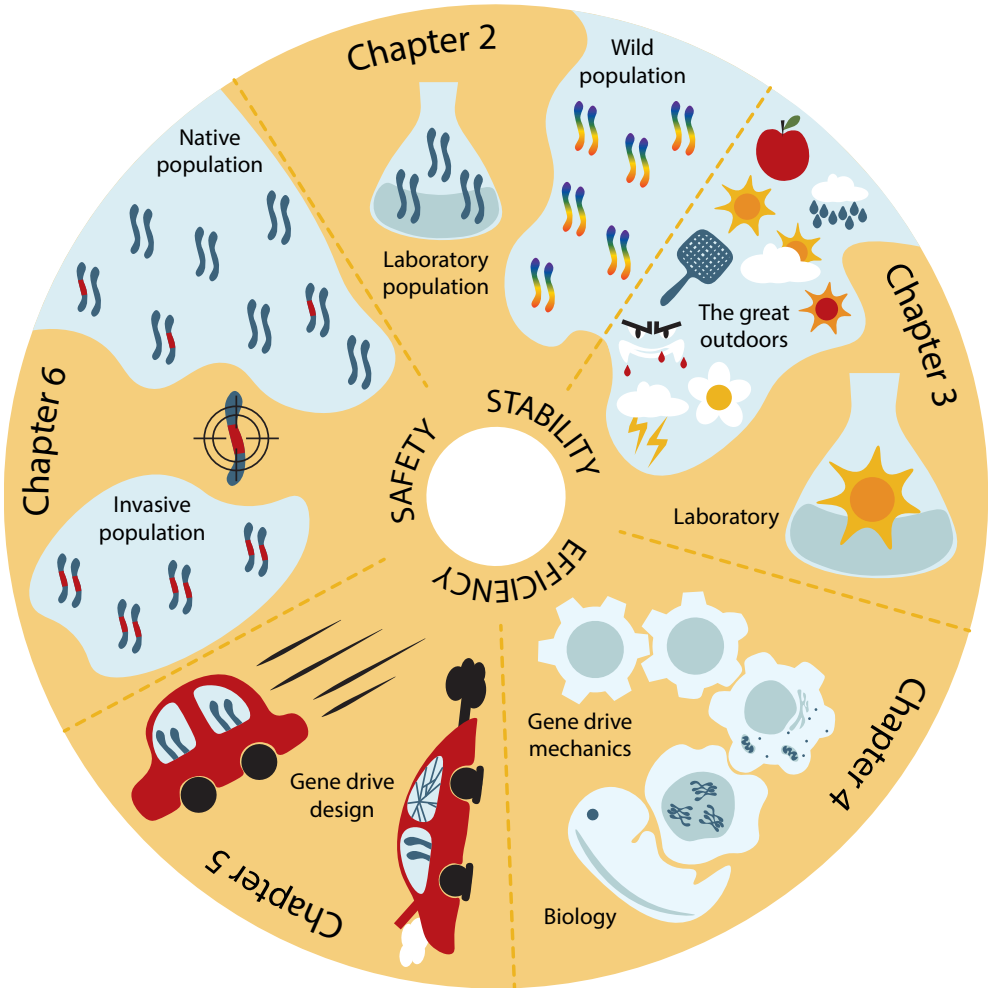


Figure 1.5. Graphical overview of this thesis.

drive intrinsic to extrinsic, and from efficiency and evolutionary stability to safeguarding (see Figure 1.5).

In **Chapter 2**, I investigate the evolutionary stability of an existing gene drive that has been highly efficient in a laboratory setting in *Drosophila melanogaster*. To this end, I look for multiple types of resistance against the drive in genetically diverse backgrounds. In **Chapter 3**, I make a first step towards elucidating the influence that a variable environment might have on the efficiency of the same gene drive, in collaboration with several BSc students. In **Chapter 4**, in collaboration with Lynn den Otter (BSc), I explore potential developmen-

tal biological explanations for the varying gene drive efficiencies that have been observed in various animal species, specifically, mice, mosquitoes, and fruit flies. In **Chapter 5**, in collaboration with Dr. Xuejiao Xu, I design an improved type of homing gene drive that can still effectively suppress a population, even when homing efficiency is low, and test it using both simulations and laboratory experiments. In **Chapter 6**, I assume that a highly efficient homing gene drive exists, and estimate the feasibility of a potential safety mechanism based on locally fixed alleles to make a gene drive spatially confined. In **Chapter 7**, I discuss my results and gene drives in a broad context.

For all the experimental work in this thesis, I have used the model species *Drosophila melanogaster*, owing to its exceptionally well-characterized genetics, which are among the most extensively studied of any animal species. Consequently, due to the wide array of available molecular techniques, *D. melanogaster* has been extensively used in gene drive research. The gene drive I used in my laboratory experiments is a modification drive, that is, one that does not impose a fitness cost on purpose and only spreads a fluorescence cargo (J. Champer, Yang, et al., 2020). I selected this type because many suppression drives still exhibit substantial fitness costs and therefore require further technical optimisation, which would obstruct the assessment of more subtle effects. However, the factors investigated here are intrinsic to all homing gene drives; consequently, the insights obtained from a modification drive are transferable to suppression drives. For the modelling parts of this thesis, I either use a generic population genetic model or a model resembling invasive mice or rats on an island.

1.5.1 Chapter 2: Investigating multiple types of resistance against a homing gene drive in European populations of *Drosophila melanogaster*

Only a few studies have investigated the influence of genetic diversity on the efficiency of different gene drives. Most notably, one study of a homing gene drive in *D. melanogaster* has found that there are significant differences between strains with different genetic backgrounds in gene drive efficiency and the rate at which resistance alleles form (J. Champer et al., 2017). These results suggest that there is a genetic component to gene drive efficiency, which might allow evolution to select for less efficient gene drive genotypes. However, another study only revealed a few loci with a small effect in a Genome-Wide Association Study (GWAS, J. Champer, Wen, et al., 2019). This absence might be because of pleiotropy, unde-

tected rare loci, too little power in the study design, or because of previously unconsidered confounding effects.

I have further explored the genetic component of drive efficiency in genetically diverse *D. melanogaster* lines collected by the DrosEU Consortium (DrosEU Consortium, 2013). This knowledge will allow us to predict how effective a gene drive will be in a genetically diverse population and to design more efficient gene drives in the future.

1.5.2 Chapter 3: Gene drive performance depends on environmentally influenced fitness factors

All gene drive studies have, to the best of my knowledge, so far only tested their gene drives in a single laboratory environment, though sometimes in large populations (Hammond, Pollegioni, et al., 2021). For *D. melanogaster*, this environment includes stable temperature, day-night cycle, nutrient availability, moisture level, and to some extent population density, among others (J. Champer, Yang, et al., 2020; Oberhofer et al., 2018; E. Yang et al., 2022). Therefore, we may have only a narrow understanding of the efficiency of many gene drive constructs. It is a well-known phenomenon that organisms become adapted to stable laboratory environments, and that specific adaptations that increase fitness in the wild are lost after some time (Hoffmann et al., 2001). Thus, a gene drive that appears efficient in the laboratory may only remain efficient if the organism is in these comfortable conditions, similar to other mutations whose effect depends partly on external conditions (Clancy et al., 2002). If harsher or more variable conditions occur in the field, it may reveal fitness costs associated with the gene drive that were not observed under laboratory conditions.

In this chapter, I take a first step in collaboration with several BSc students towards estimating the resilience of gene drive carriers in variable environments by testing a homing gene drive while altering only a single, yet important, factor relevant for *D. melanogaster*: the amount of yeast in their diet. Yeast is the main source of protein for *D. melanogaster* and influences many aspects of their life history: reproduction, lifespan, developmental time, and health, among many others (Jang & Lee, 2018; May & Zwaan, 2017; Zandveld, 2017). Varying this single factor may lead to considerable gene drive performance differences between dietary yeast concentrations.

1.5.3 Chapter 4: Why don't mice drive? A developmental biology perspective on barriers to homing gene drive efficiency

As described in detail above, homing gene drives face challenges with homing efficiency in most species (Verkuijl, 2023). Many people in the gene drive field have taken a biotechnological view of this issue when trying to improve these gene drive constructs, which can be done by using different promoters, targeting different genes, or changing other aspects of the construct (M. A. E. Anderson et al., 2023; Du et al., 2024; Verkuijl et al., 2022; Zhou et al., 2025). Although significant progress has been made this way, it also seems likely that improvements can be made more efficiently by considering the biology of target species in more detail, or that there are biological constraints on how efficient a homing gene drive can be in various species.

In this chapter, in collaboration with Lynn den Otter (BSc), I consider biological factors that might impact gene drive efficiency. As gene drive activity takes place in the germline, we investigate different developmental biology and genomic factors and discuss how they may be related to observed gene drive efficiencies in mice, mosquitoes, and fruit flies.

1.5.4 Chapter 5: Improving the suppressive power of homing gene drive by co-targeting a distant-site female fertility gene

Despite our best efforts to optimise homing gene drives, in the end, it may be that there is some unalterable biological limitation in certain species. So far, specifically in mice (and perhaps all mammals), homing efficiency has been low (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021). Therefore, we likely need gene drive designs that are robust enough to remain effective despite minor or major inefficiencies.

To this end, in this chapter I design, model, and test a novel type of homing gene drive. This gene drive partly separates the two tasks of a suppression gene drive: spreading through a population and causing a deleterious fitness effect. This separation is achieved by targeting two genes instead of one. In collaboration with Dr. Xuejiao Xu, I also test a prototype of this gene drive design in *D. melanogaster*.

1.5.5 Chapter 6: On safeguarding gene drive technology by targeting locally fixed alleles

From the very conception of CRISPR-based gene drives, targeting locally fixed alleles was considered a potentially good safety mechanism to create a so-called "precision gene drive" (Esvelt et al., 2014). A precision gene drive would only be able to spread in the target population and not in others, where the locally fixed allele is largely absent (Sudweeks et al., 2019). However, the existence of locally fixed alleles in invasive populations has been shown to be limited in small island populations of mice (Oh et al., 2021) or larger populations of invasive social wasps (Lester et al., 2020). Indeed, many invasive populations have, on an evolutionary timescale, only existed since extremely recently, mostly since ±500 years when large-scale human travel and globalisation started. This timeline likely limits the evolution of locally fixed alleles that can be targeted to create a precision gene drive, and thus the practical feasibility this safeguarding mechanism.

I first model the evolution of locally fixed alleles to estimate the likelihood that they evolve, either due to genetic drift or adaptive evolution. Second, I design a potentially more effective way to target locally fixed alleles with a gene drive that includes a self-destruct mechanism.

1.5.6 Chapter 7: General discussion

In this final chapter I discuss my results in the broader context of applying gene drive technology to manage invasive species. I discuss the results of this thesis and also discuss the gene drive field at large.

"A human being in perfection ought always to preserve a calm and peaceful mind and never to allow passion or a transitory desire to disturb his tranquility. I do not think that the pursuit of knowledge is an exception to this rule. If the study to which you apply yourself has a tendency to weaken your affections and to destroy your taste for those simple pleasures in which no alloy can possibly mix, then that study is certainly unlawful, that is to say, not befitting the human mind. If this rule were always observed; if no man allowed any pursuit whatsoever to interfere with the tranquillity of his domestic affections, Greece had not been enslaved, Caesar would have spared his country, America would have been discovered more gradually, and the empires of Mexico and Peru had not been destroyed.
But I forget that I am moralizing in the most interesting part of my tale; and your looks remind me to proceed."

— Mary Shelley, Frankenstein

"If the animals have died it will not have been quietly. It will not have been without a desperate fight. If they've died, all of them, it's because we made the world impossible for them."

— Charlotte McConaghy, Migrations

Chapter 2

Investigating multiple types of resistance against a homing gene drive in European populations of *Drosophila melanogaster*

This chapter is based on:

Faber, N. R., Champer, J., Pannebakker, B. A., Zwaan, B. J., & van den Heuvel, J. (2025). Investigating multiple types of resistance against a homing gene drive in European populations of *Drosophila melanogaster*. *bioRxiv*. https://doi.org/10.1101/2025.06.06.658298

2.1 Abstract

Gene drive technology may be a valuable tool for addressing several contemporary challenges, including combating disease vectors, conserving biodiversity, and controlling agricultural pests. Homing gene drives spread through a population by copying themselves onto the homologous chromosome in the germline of heterozygous individuals. However, resistance can evolve against homing gene drives, especially when attempting to suppress or eliminate a pest population so that resistance alleles have a large selective advantage over the gene drive. Resistance can result from a simple mutation at the drive's target site, which is found in many studies but can potentially be avoided by improving drive design. However, a more complex polygenic type of resistance could also evolve through selection on standing genetic variation that affects the efficiency of the gene drive. In this study, we test an efficient homing gene drive in genetically diverse lines of *Drosophila melanogaster*, collected from across Europe by the DrosEU Consortium. Firstly, we find that the gene drive shows considerable variability in homing efficiency, but that none of this variability can be ascribed to heritable genetic effects. Selection for complex resistance is thus unlikely and will be inefficient. Secondly, although our tested gene drive targets a highly conserved haploinsufficient gene with two gRNAs, we find two types of simple resistance alleles in viable offspring. Therefore, more effective strategies may be required to address simple resistance mutations, whereas complex resistance may be unlikely to pose a substantial barrier to the employment of at least certain types of gene drive.

2.2 Introduction

Gene drive technology could be a valuable tool for addressing several contemporary challenges, including combating disease vectors such as malaria mosquitoes, as well as conserving biodiversity and managing agricultural pests by controlling invasive species (Esvelt et al., 2014). A gene drive is a genetic element that, once released into a population, is inherited at super-Mendelian rates, thereby increasing its frequency over successive generations (Alphey et al., 2020; J. Champer et al., 2016). Synthetic gene drive systems can be designed with two purposes in mind: modification drives, which aim to spread a neutral or beneficial trait, and suppression drives, which purposefully cause a deleterious effect to shrink or eradicate populations of harmful species (Rode et al., 2019). Although the technology holds significant potential, much work remains to be done before it can feasibly be used for practical applications (Bier, 2022; Grunwald et al., 2022; McFarlane et al., 2023; Verkuijl et al., 2022). This work includes achieving the necessary high inheritance rates and high fitness of drive carriers for the gene drive to establish and spread in a population (Reid et al., 2022a; Verkuijl et al., 2022; Weitzel et al., 2021; X. Xu et al., 2022), avoiding the formation of resistance alleles that block the drive and can negate its effects (Carrami et al., 2018; J. Champer et al., 2017; Hammond et al., 2017; Pham et al., 2019), achieving localization for global biosecurity (J. Champer, Champer, et al., 2021; Dhole et al., 2018, 2019; Noble et al., 2019), as well as establishing regulatory frameworks for the governance of this technology, alongside engaging the general public (de Graeff et al., 2018; National Academies of Sciences, Engineering, and Medicine, 2016).

The evolution of resistance presents a significant obstacle, because it undermines the gene drive's intended purpose either partially or entirely (Price et al., 2020). Especially in the case of suppression drives, resistance is likely to pose a problem due to the large selective advantage that resistant individuals will have once the population is reduced in size. Resistance can take three general forms (Price et al., 2020), the first of which is through mutations in the gene drive's target site, which have been found in many experimental demonstrations of gene drive constructs (Carrami et al., 2018; J. Champer et al., 2017; Hammond et al., 2017; Pham et al., 2019). We call this type of resistance "simple resistance" (Figure 2.1). The mutations at the target site usually arise through the drive's activity itself (J. Champer et al., 2017). However, they can also already be present in a population, and requires screening of the target population beforehand (Drury et al., 2017; Oh et al., 2021; Willis & Burt, 2021).

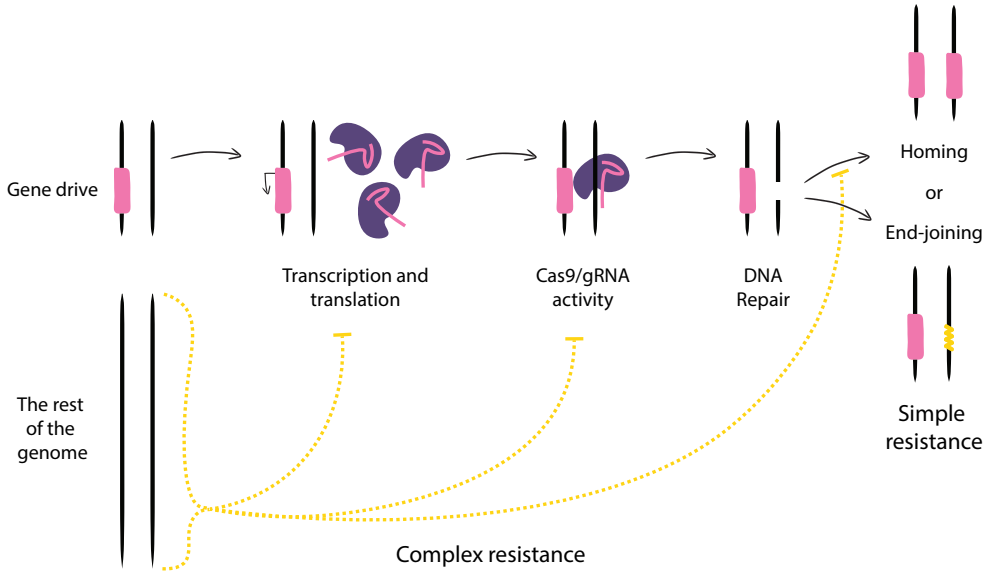


Figure 2.1. Schematic of simple and complex resistance against a CRISPR-based homing gene drive. Pink and purple show the gene drive components going through the various steps of activity, with the outcome being either homing or end-joining. Yellow lines represent resistance against the gene drive, with the squiggly yellow line representing simple resistance after end-joining repair (instead of homology-directed repair for homing), and the arrows inhibiting different processes indicating complex resistance. We show three processes essential for homing in which complex genomic resistance could theoretically interfere, but there could be many others.

This type of simple resistance seems largely avoidable by good gene drive design, such as targeting conserved sequences and gRNA multiplexing (M. A. E. Anderson et al., 2024; S. E. Champer et al., 2020; Chen et al., 2024; Edgington et al., 2020; Faber et al., 2024; Kyrou et al., 2018; Willis & Burt, 2021; E. Yang et al., 2022). The other two ways in which resistance could evolve as described by Price et al. (2020) are through molecular interference from non-target genetic elements, and through behavioural or life history changes within the population. As these types of resistance are likely to be complex, quantitative traits, we call them "complex resistance" hereafter (Figure 2.1).

The forms of complex resistance have remained mostly theoretical (Verma et al., 2022)

and most gene drives are only tested in single laboratory strains, such as the white-eyed *Drosophila melanogaster* strain w^{1118} (Du et al., 2024; Gantz & Bier, 2015; López Del Amo, Bishop, et al., 2020; Oberhofer et al., 2018). Although there have been a few experimental studies testing a gene drive in different genetic backgrounds, none of them have formally calculated the heritability of their efficiency, nor accounted for confounding factors such as maternal, environmental, or random effects (J. Champer, Wen, et al., 2019; J. Champer et al., 2017; DiCarlo et al., 2015; Hernandes et al., 2024; Oberhofer et al., 2019; Pescod et al., 2023). Broadly speaking, evolutionary history has demonstrated that resistance can arise against many ecological interventions when there is a significant enough benefit to it (Bark, 2010; Christiaens et al., 2022; De Rouck et al., 2023; Ffrench-Constant, 2013; McGee et al., 2020; Peterson et al., 2018; Yin et al., 2023). Also, in theory, there are many molecular processes essential for gene drive functioning for which standing genetic variation may be present in the targeted population and that could convey (partial) complex resistance. In the case of CRISPR-based homing gene drives, which we focus on in this study, Cas9 cutting efficiency could be reduced, DNA repair could switch from homology-directed repair to end-joining, the timing of the expression of the drive may be shifted, the deleterious effect that the gene drive causes could be mitigated, or the recessive genetic load that the drive imposes may not stay fully recessive, among many other potential mechanisms. Thus, anything the gene drive field is currently trying to optimize in their constructs could be counteracted by the evolution of complex resistance (Grunwald et al., 2022; Verkuijl et al., 2022). Exploring the likelihood that complex forms of resistance arise is important for estimating the evolutionary hurdles that gene drive technology may yet face, especially as some drive constructs may be ready for field trials in the near future (Connolly et al., 2024; Hammond, Pollegioni, et al., 2021; Long et al., 2020).

Besides simple and complex resistance, there is an additional type of resistance to consider for a specific type of homing gene drive: the homing rescue drive. Some modification gene drives (which aim to drive a trait into a population, instead of to suppress it) are located within a gene while also containing a recoded "rescue" element for this gene. The rescue element needs to be recoded with synonymous substitutions to avoid cleavage by the gene drive's gRNAs. This construction allows the gene drive to avoid simple resistance by targeting highly conserved genes, because most simple resistance alleles will be deleterious, whereas the gene drive with the rescue element will be fully functional. However, it also puts the gene drive at risk of partial homing, where homology-directed repair could, after

copying the rescue of the gene drive, revert back to the wild-type strand without copying the other components of the gene drive. The recoded rescue element still has some homology with the wild-type sequence (usually including part of the 3' untranslated region of the gene as well), which could facilitate this partial homing. The result will essentially be a simple resistance allele, only it is more likely to be a functional resistance allele than end-joining mutations (which are often out-of-frame). However, this mechanism has not yet been demonstrated in practice for any homing rescue gene drive (Adolfi et al., 2020; J. Champer, Yang, et al., 2020; Hou et al., 2024; Kandul et al., 2021; Terradas et al., 2021).

In this study, we test all these different types of resistance that could evolve against a homing rescue gene drive (J. Champer, Yang, et al., 2020). We investigate the impact of standing genetic variation on gene drive efficiency to estimate the likelihood that complex resistance could pose a problem for gene drives in genetically diverse populations in the field. To this end, we test the gene drive in a broad range of genetically diverse *Drosophila melanogaster* populations. These populations were sampled by the DrosEU consortium from across Europe (Kapun et al., 2020), and isofemale lines were created as a snapshot that captures the genetic diversity in these populations at the time (David et al., 2005). Specifically, we measure the two main phenotypes indicative of gene drive success to cast as wide a net as possible: gene drive inheritance rate and number of offspring. We find that the gene drive shows considerable variability in inheritance rates, but this variability cannot be ascribed to heritable genetic effects. Also, there is a notable shift in the number of offspring produced between gene drive and control crosses, but again, this shift cannot be linked to the genetic background of the cross. Finally, from crosses that exhibited especially low efficiency, we screened viable offspring for resistance alleles at the gRNA target sites, and found both deletions and partial homing events. We conclude that simple resistance is more likely to evolve than complex resistance, and we suggest that the modularity of gene drive technology can be harnessed to manage this.

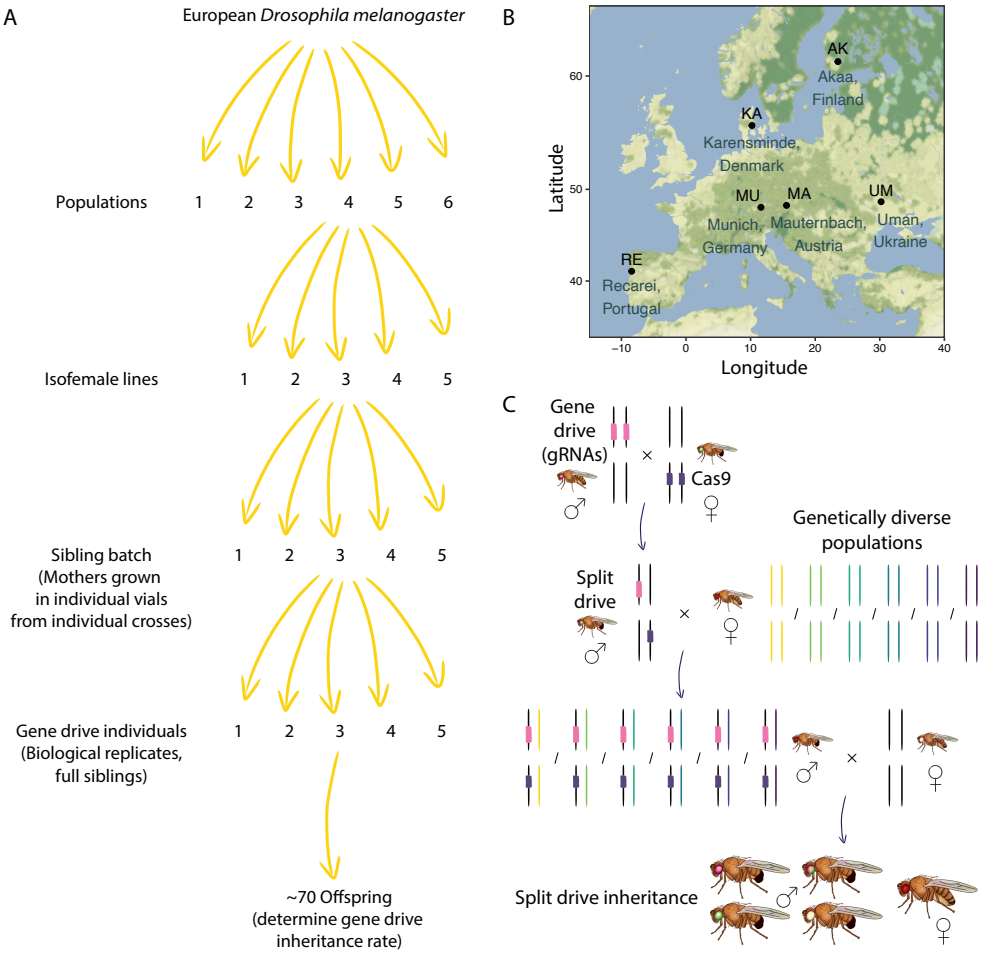


Figure 2.2. Experimental design to determine the heritability of gene drive efficiency. A) We use a nested hierarchical experimental design with four levels: population, isofemale line, sibling batch, and finally, gene drive individual. For illustrative purposes, arrows for the next level down are only drawn for one replicate. B) Geographic locations of the six (out of nine in total) *D. melanogaster* populations that were sampled by the DrosEU Consortium and used in this study. C) A simplified version of our split homing rescue gene drive crossing scheme.

2.3 Methods

2.3.1 Nested full-sibling crosses to estimate heritability

To calculate the heritability of gene drive efficiency, we use a nested full-sibling experimental design. We have three levels of nesting, namely population, isofemale line, and sibling batch (Figure 2.2A). We tested six populations, five isofemale lines from each population, and five sibling batches per isofemale line, with five gene drive males from each batch as biological replicates (full siblings). We only test the heritability of homing efficiency in male drive individuals, because our chosen Cas9 is predominantly active in the male germline. We use a Generalized Linear Mixed Effect model to partition the observed variance to each level in our experiment (Brooks et al., 2017). We consider population-level and isofemale-level effects as genetic effects, whereas the sibling batch is used to correct for potential maternal or environmental effects, and between-sibling variance indicates stochastic effects. We note that there may still be genetic variation within each isofemale line, as they were not inbred upon collection. Therefore, our heritability estimate will be conservative. We calculate the heritability as follows:

$$H^2 = \frac{Var(\text{population}) + Var(\text{isofemale line})}{Var(\text{total}) + \pi^2/3}, \tag{2.1}$$

where $\pi^2/3$ is the variance of binomially distributed data (Nakagawa & Schielzeth, 2010).

2.3.2 Choice of genetically diverse *Drosophila melanogaster* lines

The genetically diverse populations we tested were sampled from across Europe by the DrosEU Consortium (Kapun et al., 2020). This consortium sampled from nine populations up to 20 isofemale lines each. We chose six populations from across the East and West populations in which European *D. melanogaster* populations are genetically structured to maximize the amount of genetic variation covered by our experiment (Figure 2.2B) (Kapun et al., 2020). Among all DrosEU populations, the pairwise F_{ST} ranges between 0.013 and 0.059, so our chosen populations cover the higher end of that range (Kapun et al., 2020). These lines were kept in laboratory conditions for several years, and some adaptation to the lab conditions is expected. While this generally deemed undesirable to get a realistic measurement of a trait relevant in the field (David et al., 2005), here we are inter-

ested in estimating the heritability of gene drive efficiency in general and not so much in whether the underlying genetic variation closely resembles that of the natural source populations.

2.3.3 Choice of gene drive and Cas9

For this study, we used a homing rescue modification gene drive previously built by J. Champer, Yang, et al. (2020). This gene drive is highly efficient and able to quickly spread through a cage population of *D. melanogaster*. The gene drive is located in the highly conserved haplolethal essential gene *RpL35A*, which encodes for a ribosomal subunit that is expressed at every life stage (Brown et al., 2014) and in every tissue (H. Li et al., 2022) of *D. melanogaster*. The gene drive targets exon 4 of *RpL35A* gene with two gRNAs, while also, after inserting itself at this location, providing a rescue for the rest of the gene, thus making it a modification gene drive. It should be noted that *RpL35A* is a "*Minute*" locus, meaning that it is haploinsufficient (Cook et al., 2012; Marygold et al., 2007), and is presumed to be a haplolethal gene (J. Champer, Yang, et al., 2020). Therefore, most or all simple resistance alleles, either from the germline itself or in the embryo from maternal deposition of Cas9 and gRNAs, result in non-viable offspring as there will be still one malfunctioning copy of *RpL35A* present after the mutational event.

This drive is also a split drive for the molecular safeguarding of experiments (Akbari et al., 2015; J. Champer, Chung, et al., 2019), which gave us the opportunity to choose our Cas9 line too. Under the *nanos* promoter, this gene drive was inherited in around 90% of offspring, but there were medium fitness costs for female carriers, as some offspring was not viable due to the above-mentioned maternal deposition of gene drive components (J. Champer, Yang, et al., 2020). For this study, we thus chose to use a different Cas9 line, namely one that was shown to be highly efficient in males and which showed a lower fitness cost in females (Du et al., 2024). This Cas9 is expressed by the *CG4415* promoter (which normally promotes the *gNacα* gene, which is a ribosome associated protein that is predominantly expressed in the male germline, Kogan et al., 2022), combined with a *nanos* 3'UTR. Both the gene drive and Cas9 constructs are contained within white-eye w^{1118} lines of *D. melanogaster* called AHDr352v2 and SNc9XnGr, respectively.

2.3.4 Experimental crosses

We follow a standard gene drive crossing scheme with an extra step to introduce genetic variation into the cross, similar to J. Champer, Wen, et al. (2019) (simplified in Figure 2.2C). In the first cross, we crossed the two lines containing the split drive elements, the Cas9 construct (with green fluorescence) and the driving construct containing the gRNAs (with red fluorescence), hereafter called the gene drive. The gene drive was contributed by the paternal side of this cross. Secondly, we crossed males heterozygous for both elements of the split drive back to females from the Cas9 line. From those offspring, we selected males carrying the gene drive (red fluorescence) and that were likely homozygous for Cas9 (bright green fluorescence rather than fainter green). Thirdly, we crossed these to females of the genetically diverse DrosEU populations. These DrosEU females were all isolated from separate single crosses in separate vials, making it possible to correct for non-genetic confounding factors such as shared environmental effects or maternal effects (Lynch & Walsh, 1998; Zaitlen & Kraft, 2012). From that offspring, we selected five males for the final cross back to w^{1118} females. These males were likely carrying the gene drive (due to it being driven in their male parent) as well as Cas9 (due to their male parent being homozygous for Cas9). Females were allowed to lay eggs for 7 days. In the offspring, all males had a white-eye phenotype (as the white-eye mutation is X-linked), making it possible to score fluorescence and calculate drive inheritance. Females had wild-type eyes, making fluorescence scoring substantially more difficult. Thus, fluorescence was only scored in male progeny. All offspring, including females, were counted.

Because we were interested in fitness costs due to the gene drive and its activity, besides these gene drive crosses we followed the same crossing scheme with flies that carried Cas9, but not the drive. This allowed us to distinguish any drive-related fitness costs from any DrosEU-w^{1118} outbreeding fitness costs.

2.3.5 Fly rearing

All crosses except the final cross were performed in bottles with 50 mL of standard medium containing yeast (70 gr/L), sugar (100 gr/L), and agar (20 gr/L), with nipagin (15 mL of 100 gram/L EtOH) and propionic acid (3 mL of 13.39 M) to avoid bacterial or fungal contamination. The final crosses were performed in vials with 6 mL of the above-mentioned medium. Flies were kept at 25°C on a 16:8 hours cycle of light:dark. Handling of flies hap-

pened under CO_2 sedation. Experiments were done in compliance with Dutch genetically modified insect regulations.

2.3.6 GLMM and data analysis

For data visualisation and data analysis, we used R version 4.4.0 (R Core Team, 2025). For the GLMM variance partitioning as well as data simulation from the fitted model, we used the package glmmTMB (version 1.1.10) (McGillycuddy et al., 2025) in combination with the DHARMa package (version (0.4.7) (Hartig, 2017) to test for uniformity of distribution. For details and code, see the Github repository (https://github.com/NickyFaber/HomingDrive_Heritability).

2.3.7 Mutations in *RpL35A* screening

To screen for mutations in *RpL35A* at the gRNA target sites, DNA was isolated from individual flies that did not inherit the gene drive (that is, did not show red fluorescence). Using a high salt protocol, flies were ground in 100 ul buffer (0.1 M Tris HCl pH 8, 1 mM EDTA, 0.25 M NaCl, 1 µg/ul Proteinase K), and incubated overnight at 55 °C. Proteinase K was inactivated by heating for two minutes at 95 °C, and tubes were centrifuged for three minutes at 15.000 rpm to collect debris at the bottom. Supernatant containing the DNA was transferred to a clean tube and diluted 10x. Primers were designed with Primer3 (Untergasser et al., 2012) and tested both on w^{1118} flies and on several wildtype DrosEU flies to check that they worked equally efficiently for both. Primer sequences are 5'GGACGCCTCTTCGCCAAG'3 for the forward primer and 5'GCAAGAAGAAAGAAGGCATGT'3 for the reverse primer. PCR was done using a standard GoTaq polymerase PCR protocol and put on a 1% agarose gel with ethidium bromide. Sanger sequencing was performed by Eurofins to determine heterozygosity. PCR product of identified heterozygous flies was sequenced with Nanopore sequencing technology by Plasmidsaurus.

Sequences were mapped to the *D. melanogaster* reference genome using Minimap2 (H. Li, 2018), after which Freebayes was used to call variants (Garrison & Marth, 2012), Whatshap was used for phasing the two haplotypes (Martin et al., 2016), and Bcftools was used to create consensus haplotypes (H. Li, 2011). A multiple-sequence alignment of the resulting haplotypes was made using the Muscle algorithm in R (version 4.4.0) with the 'msa' package (version 1.38.0) (Bodenhofer et al., 2015). All programs were ran with default pa-

rameters, but for data and code, see Github repository (https://github.com/NickyFaber/HomingDrive_Heritability).

To check for standing genetic variation at the *RpL35A* gRNA target loci in the DrosEU populations, we used the available DrosEU vcf file containing variant loci and allele frequencies per population (Kapun et al., 2020).

2.4 Results

In total, our experiment consisted of 1343 crosses, 656 of which were gene drive males crosses with w^{1118} females, and 687 of which were Cas9-only males crossed with w^{1118} females as control. Fluorescence was scored in all male offspring of the gene drive crosses (female offspring had red wild-type eyes that makes fluorescence scoring difficult, see Figure 2.2C), and for both the gene drive and control crosses the number of offspring was counted. Finally, 63 offspring from the 30 gene drive crosses with the lowest gene drive inheritance rates were sequenced to identify mutations that are potential simple resistance alleles.

2.4.1 Gene drive inheritance rate

The first high-level phenotype that reflects gene drive efficiency is gene drive inheritance. Plotting the gene drive inheritance rate in male heterozygotes, the most visually striking result is how similarly the gene drive performs across all groups (Figure 2.3 for gene drive inheritance and Figure S2.1 for Cas9 inheritance). The absence of a pattern is also clear when plotting the data only by population (Figure S2.2) or isofemale line (Figure S2.3). Because there is a significant amount of spread in the number of offspring produced in each cross (see next section), we confirmed that gene drive inheritance is not meaningfully impacted by this due to, for example, larval crowding effects (Figure S2.4 and Figure S2.5).

Fitting a GLMM containing the three experimental hierarchical levels (population, isofemale line, and sibling batch) as nested random effects, we find that there are significant problems with uniformity of distribution (Asymptotic one-sample Kolmogorov-Smirnov test, $p = 0.0027$), dispersion (DHARMa nonparametric dispersion test, $p = 0.04$), and outliers (DHARMa bootstrapped outlier test, $p = 0.016$). This means that our data significantly diverges from a true binomial distribution due to unaccounted causes of variance.

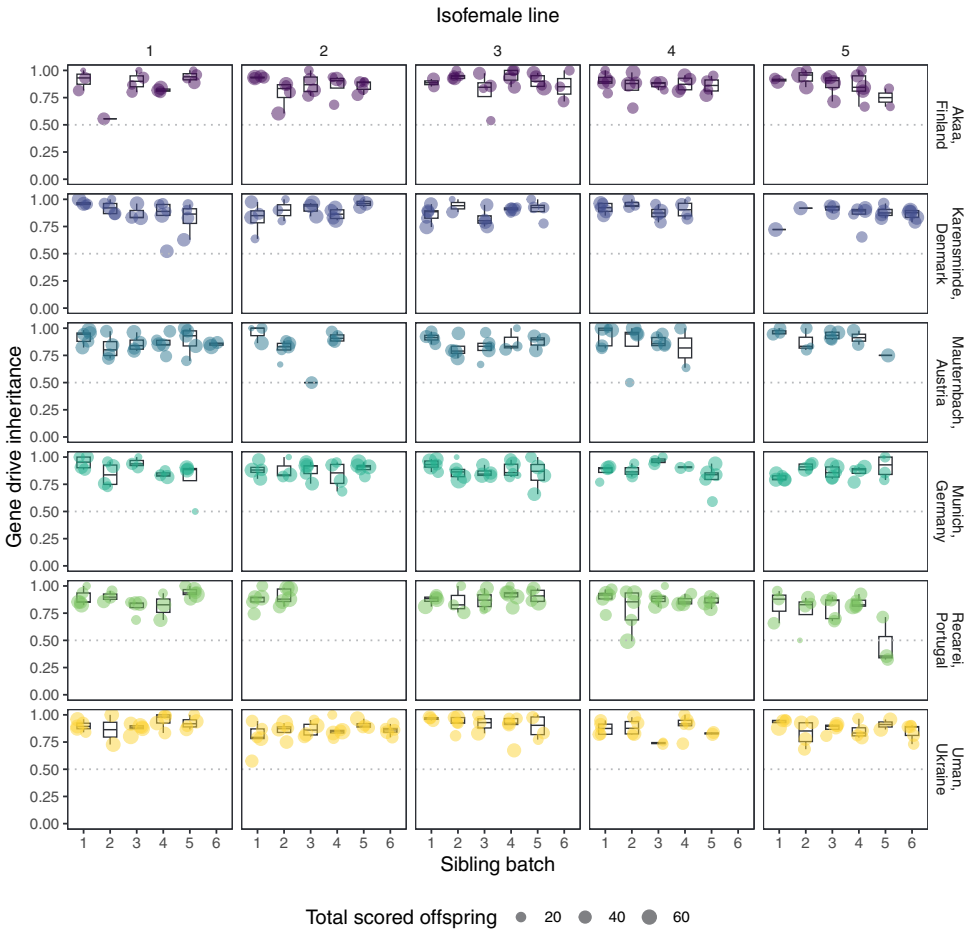


Figure 2.3. Gene drive inheritance rates plotted per each hierarchical level of the experiment. A dashed line at 0.5 indicates Mendelian inheritance. The size of each dot indicates the number of male offspring in that cross. In total, out of 656 crosses that were made, 579 crosses produced at least one male offspring that could be scored for fluorescence. For the same plot grouped only by higher levels in our experiment (population and isofemale line), see Figure S2.2 and Figure S2.3.

We visualize this in Figure S2.6, where we show the distribution of our observed data *versus* 100 simulations of a true binomial distribution from the fitted model. To account for the unknown causes of variance in our experiment, we add an observation-level random effect to the model.

After performing model selection by sequentially removing the hierarchical level that explains the least amount of variance, we conclude that the best fitting model keeps only the lowest level (the observation-level random effect, cross). This means that none of the hierarchical levels in our experiment significantly contributes to explaining the variance observed in gene drive inheritance rates. Neither sibling batch ($\chi^2(1) = 2.23, p = 0.14$), isofemale line ($\chi^2(1) = 0.95, p = 0.33$), or population ($\chi^2(1) = 0.00, p = 1.00$) are kept as random effects, nor who (out of four people) scored the flies as a fixed effect ($\chi^2(3) = 3.50, p = 0.32$) (Table S2.1). From the model containing all four experimental levels, the total amount of variance that can be explained by each level is shown in Figure 2.4, with effect ranges per group shown in Figure S2.7. Using the heritability formula specified in Equation 2.1, we calculate that the heritability of this gene drive's efficiency (that is, inheritance rate) in the DrosEU lines is 0.31%, though this value is likely not significantly different from 0% as the best fitting model retains neither of the two experimental levels representing genetic effects.

2.4.2 Number of offspring

The second factor determining gene drive efficiency is fitness of gene drive carriers, which we measure here as the total number of adult offspring. Because *RpL35A* is a haplolethal gene, any offspring inheriting a non-functional allele due to indels will likely not be viable (J. Champer, Yang, et al., 2020). Furthermore, just the presence of active Cas9 could lead to a fitness costs, likely due to off-target cutting (Langmüller et al., 2022). When plotting the overall number of offspring from gene drive crosses and control crosses (which only carried Cas9, and not the gene drive itself), we see that the average number of offspring is similar between gene drive crosses (mean = 68.15, n = 656) and control crosses (mean = 68.89, n = 687). However, the distribution appears wider in both directions for gene drive crosses, indicative of the gene drive causing some fitness effects (Figure 2.5). When plotting the data per population (Figure S2.8), per isofemale line (Figure S2.9), or per sibling batch (Figure S2.10), subtle differences in the number of offspring between isofemale lines are

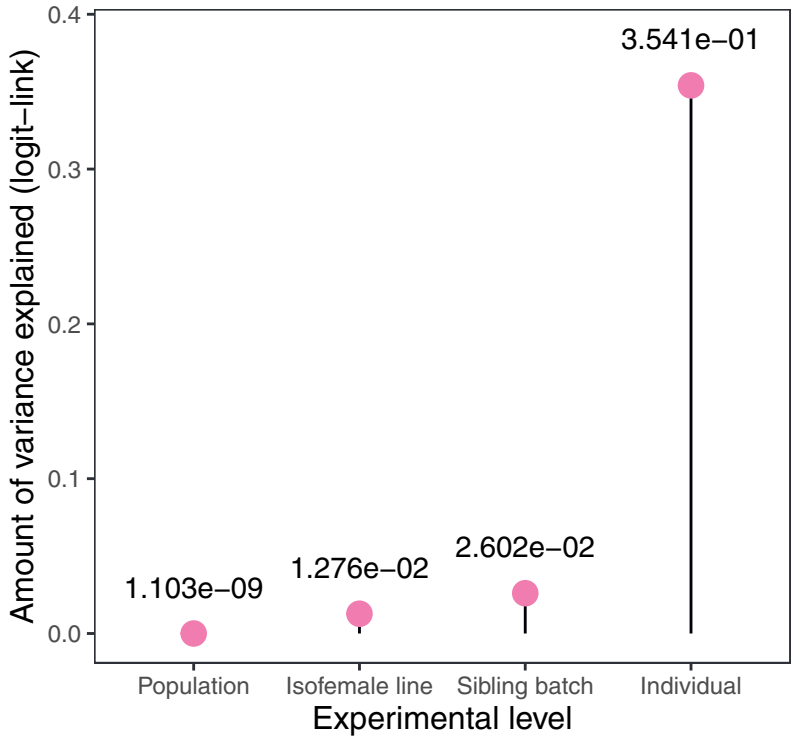


Figure 2.4. Variance in gene drive inheritance rate partitioned to each hierarchical level of our experiment. These values are logit-linked.

visible, but there is a large amount of overall variance.

Fitting a GLMM that corrects for zero-inflation (as some crosses produced no offspring), we find that a Gaussian distribution with a zero-inflation model fits our data the best (Figure S2.11). The full model contains the three experimental hierarchical levels (population, isofemale line, and sibling batch) as nested random effects, and genotype (gene drive or control) as a fixed effect, as well as interactions between genotype and population or line. This interaction indicated any gene drive effect that is specific to a population or line. The difference in heterogeneity of variance between control and gene drive crosses is significant (Levene's Test for Homogeneity of Variance, $p = 0.032$) (Figure 2.5). Otherwise, the distribution of the data is normal in terms of uniformity (Asymptotic one-sample Kolmogorov-Smirnov test, $p = 0.26$) and dispersion (DHARMa nonparametric dispersion test, $p = 0.8$), although there are fewer outliers than expected, probably due to the zero-inflation

model (DHARMa bootstrapped outlier test, p < 0.001). We do model selection by sequentially removing the effect that explains the least amount of variance. The best fitting model contained only isofemale line ($\chi^2(1) = 45.60, p < 0.001$), but not population ($\chi^2(1) = 0.57, p = 0.45$), or any of the interactions between population and isofemale line (see Table S2.2 and Figure S2.12). Isofemale line is expected to be a significant factor, as setting up isofemale lines creates significant genetic bottlenecks (David et al., 2005). Overall, we conclude that there is no meaningful interaction between genetic background and the fitness effect of the gene drive (measured as the total number of offspring). However, due to the large amount of total variance we observe, perhaps a more targetted experimental set-up than the one used in this study is necessary to uncover potential small effects.

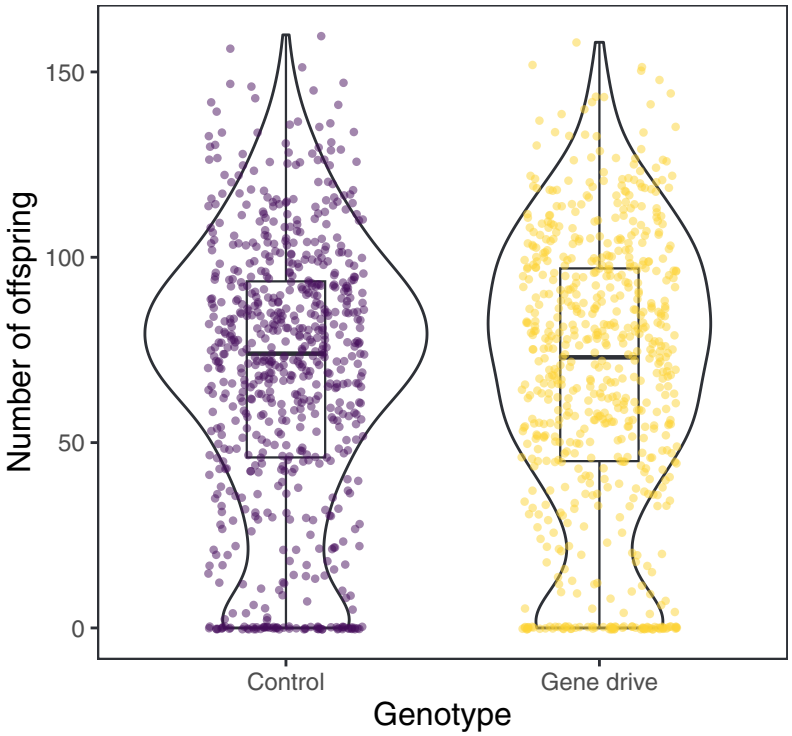


Figure 2.5. Number of offspring from gene drive and control crosses. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. The data is additionally plotted per population in Figure S2.8, per isofemale line in Figure S2.9, and per sibling batch in Figure S2.10.

2.4.3 Resistance alleles in RpL35A at the gRNA target sites

We investigated the crosses with the lowest gene drive inheritance for potential simple resistance alleles. These alleles could already have been present in the DrosEU populations, or they could have been formed through gene drive activity. Based on population genetic data from the DrosEU consortium, we do not expect any standing variation as there is not a single variant observed at either of the two gRNA sites in any population across Europe (Figure S2.13A and B). Furthermore, plotting the frequency of variants in the whole *RpL35A* gene, we see that most variants are at low frequency in most populations (Figure S2.13C). Therefore, variants in the homology regions during gene drive homing seemed unlikely, but not an impossible, cause for the occasionally strongly reduced homing efficiencies (Ang et al., 2022; Do et al., 2014). At the same time, we assumed resistance allele formation through gene drive activity itself to be highly unlikely too due to the conserved and haplolethal nature of the RpL35A target gene, together with the use of two gRNAs (J. Champer, Yang, et al., 2020).

From each of the 30 lowest-drive inheritance crosses, we took up to three males that did not inherit the gene drive (that is, they were not red fluorescent), and amplified and sequenced roughly 400 bp of the *RpL35A* locus (Figure S2.13A). Surprisingly, out of 63 flies in total, 11 flies were heterozygous at the *RpL35A* locus, whereas the rest was homozygous wildtype (Figure 2.6). The resistance alleles were never the same between siblings of a single cross, and were also not disproportionally found in any particular population or isofemale line. These 11 resistance alleles can be classified into two types: 1) double deletions at both gRNA sites and 2) partial homology-directed repair from the gene drive's *RpL35A* rescue element. Even more surprisingly, out of the six haplotypes with double deletions, only three leave *RpL35A* in frame, and the other three haplotypes have a frame-shift in *RpL35A*. Although *RpL35A* is assumed to be a haplolethal gene, these flies were viable despite this frame-shift, because they lived to adulthood before they were frozen at the end of the experiment. Because of this, the fertility of these flies could not be checked and we cannot say whether they are functional resistance alleles (that is, potentially transmitted to the next generation).

Of the five haplotypes that showed partial homology-directed repair from the drive allele (as can be seen by the substitution roughly every third base, which was designed to give drive individuals a functioning *RpL35A* rescue, without it being susceptible to cutting by

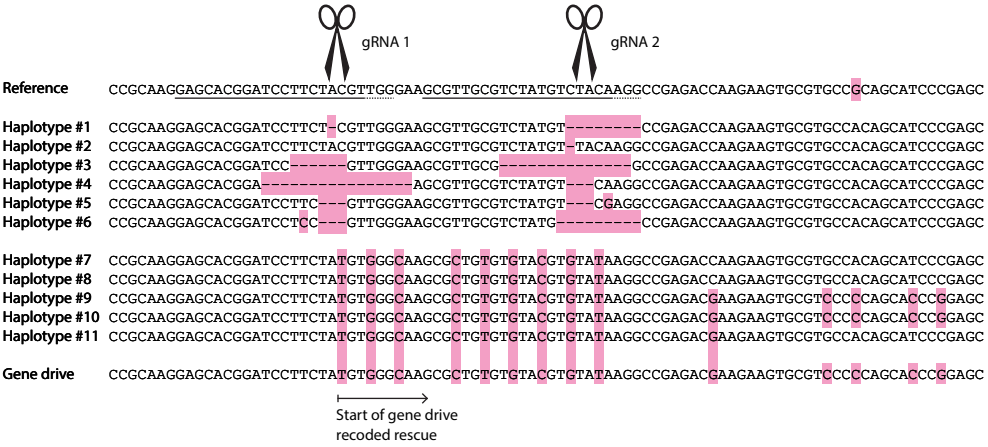


Figure 2.6. Multiple sequence alignment of the 11 observed RpL35A resistance alleles. On top is the *D. melanogaster* reference genome, where we show the gene drive's two gRNAs targets (solid underscore indicates the 20 bp gRNA target sequence, dashed underscore indicates the PAM-sequence, and scissors indicate the location where the double-stranded break will occur. Below the reference sequence, we show the 11 resistance allele haplotypes that were found, with pink blocks indicating deletions and mismatches with the reference. At the bottom, we show the gene drive sequence. Note that haplotypes number 9 and 10 contain a slightly larger piece of the recoded sequence than is shown here (Figure S2.14); for the full alignment, see the GitHub repository (https://github.com/NickyFaber/HomingDrive_Heritability).

the gRNAs), each showed varying lengths of the recoded part of the gene drive (Figure S2.14); see the GitHub repository for the full alignment, https://github.com/NickyFaber/HomingDrive_Heritability. Of these, we also cannot conclusively say that they are functional resistance alleles, although arguably these are highly likely to be functional *RpL35A* copies as gene drive individuals are fully fertile with the entire recoded sequence. These genotypes are also likely not susceptible to cutting by the gRNAs because they contain several mismatches at the PAM-proximal end, where mismatches are less tolerated (Hsu et al., 2013). These data show that viable resistance alleles are possible in conserved genes such as *RpL35A*. All in all, this is the first demonstration that partial homology-directed repair could facilitate simple resistance allele formation for gene drives containing a recoded rescue element.

2.5 Discussion

As with any biocontrol method, it is crucial to anticipate the evolution of resistance—regardless of its form—and develop appropriate mitigation strategies (Bark, 2010; Christiaens et al., 2022; De Rouck et al., 2023; Ffrench-Constant, 2013; McGee et al., 2020; Peterson et al., 2018; Yin et al., 2023). The well-documented and expected form of resistance against gene drives arises from mutations at its gRNA target sites, which are highly likely to occur and necessitate countermeasures (Carrami et al., 2018; J. Champer et al., 2017; Hammond et al., 2017; Pham et al., 2019; Price et al., 2020). In contrast, complex forms of resistance remain understudied and their potential impact on gene drive efficacy is not yet well understood (J. Champer, Wen, et al., 2019; Price et al., 2020). In this study, we investigate the potential for complex resistance to emerge against a CRISPR-based homing gene drive. We do so by testing a modification rescue drive in diverse genetic backgrounds in a nested full-sibling design to estimate heritability, while accounting for non-genetic confounding factors (Lynch & Walsh, 1998). We assessed two key indicators of gene drive efficiency: the inheritance rate of a homing rescue gene drive and the fitness of its carriers as the total number of offspring. Additionally, we screened for mutations that could indicate the presence of simple resistance alleles.

Our findings reveal an exceptionally low heritability, 0.31%, for the inheritance rate of the homing rescue gene drive in male carriers—far lower than typical heritable traits in *D. melanogaster* (Roff & Mousseau, 1987). Several factors may be behind this low heritability. Firstly, although intuitively it makes sense to think of gene drive efficiency as having a genetic component, the gene drive may just be a mostly self-contained construct that relies on gene expression machinery. Indeed, different Cas9 promoters show more significant variation in gene drive inheritance rates than we observed here (M. A. E. Anderson et al., 2023; Du et al., 2024). Secondly, perhaps genetic effects are so small that our experimental set-up is not sensitive enough to pick them up. As complex cellular mechanisms underlie gene drive efficiency (Cas9 expression, Cas9 protein activity, DNA accessibility, and DNA repair mechanisms), this seems possible (J. Champer et al., 2017; Verkuijl et al., 2022). Thirdly, these mechanisms may be highly conserved, thus limiting variation. However, there is evidence for genetically determined variation in somatic DNA repair, indicating that this might be possible in the germline too (Svetec et al., 2016). Finally, potential trade-offs between DNA repair pathways and fitness could constrain observable differences. For

example, a shift from homology-directed repair to end-joining repair in the germline could lead to a direct fitness cost because of the necessary double-stranded break repairs during crossing-over, thus still keeping overall drive inheritance similar by removing wild-type alleles instead of turning them into drive alleles.

The second part of gene drive efficiency we assessed was the total number of offspring. End-joining could lead to lethal genotypes in the offspring, an effect that would be visible as a reduction in offspring numbers. Although we saw a noticeable, though small, shift in the distribution, this effect was likely confounded by larval crowding effects and thus hard to statistically analyse. Further, fecundity is a highly variable trait in *D. melanogaster* (Robertson, 1957), so observing a reduction in the number of offspring is harder to measure than the occurrence of a visible phenotype such as fluorescence or a yellow body colour (J. Champer et al., 2017).

The low observed heritability, despite substantial observed variation, suggests that selection for complex resistance against this gene drive in genetically diverse populations will be inefficient. This is beneficial for gene drive efficacy, as it implies this gene drive will likely remain relatively stable over generations. However, with a large enough selection pressure, for example, if this gene drive were converted to a suppression drive (Faber et al., 2024), and a large enough population, complex resistance could still be selected for. Namely, we observed a considerable amount of variation in drive efficiency, and variability and heritability together determine the evolvability of a quantitative trait (Houle, 1992). Before field trials are initiated, it is essential to screen gene drives specifically in the genetic background of the target population to better understand the potential for complex resistance across different constructs, populations, and species. While field trials could reveal the effects of complex resistance, they also carry the risk of irreversibly altering the genetic makeup of wild populations. Although our findings suggest that selection for complex resistance is likely inefficient, resistance mechanisms such as Cas9 inhibition or shifts in DNA repair pathways would impact all gene drives relying on these processes. Unlike simple resistance, these forms of resistance cannot always be mitigated by modifying gRNAs or designing a new gene drive (Edgington et al., 2020). This consideration should be incorporated into risk assessments and public engagement efforts prior to field deployment. Regular sampling of heterozygous gene drive individuals from genetically diverse populations and testing their gene drive efficiency in the lab could provide ongoing monitoring

of drive functioning.

Although our primary focus was to investigate complex resistance, we also identified multiple resistance alleles at the gRNA target sites in *RpL35A*. These resistance alleles were somewhat unexpected given the conserved nature of this gene, which codes for a ribosomal subunit that was believed to be haplolethal, though mosaic resistance was seen previously (J. Champer, Yang, et al., 2020) (see previous modelling of partial repair, S. E. Champer et al., 2020). Based on the mutations we found in *RpL35A*, some of which were out of frame, it seems likely that this gene is not completely haploinsufficient, perhaps at least allowing some mutations in some genetic backgrounds to be viable (Cook et al., 2012). Although we cannot determine whether these mutations confer functional simple resistance, it could be that they are at least recessively lethal. However, we believe that the resistance alleles copying some of the recoded part of the gene drive (replacing the original wild-type sequence) may represent functional resistance as gene drive individuals are also fully viable and fertile with the full recoded sequence.

These findings raise a key question: will gene drive resistance in natural populations primarily arise as simple mutations, complex mechanisms, or perhaps not at all? Our data suggest that simple resistance will be the more immediate concern, and that homing rescue gene drives have an additional mechanism through which they are vulnerable to the formation of these alleles. It is not immediately clear how these alleles can be mitigated. One option is through multiplexing with more than two gRNAs, as modelled by S. E. Champer et al. (2020). Another option is to target conserved genes with less functional redundancy than RpL35A such as *doublesex* (Chen et al., 2024). In any case, if this gene drive were converted to a suppression drive, this type of two-target suppression drive is more tolerant of simple resistance alleles at the homing site, as the genetic load is mostly imposed at the distant target (Faber et al., 2024). Adjusting the 3' UTR to a version from another species (with several mutations to break up the large tract of identical DNA) could both improve drive efficiency and reduce the chance of partial homology-directed repair (though it is unclear to what extent the 3' UTR is involved in the partial homing process), though it might also reduce the chance of successful rescue (Hou et al., 2024). Using genes with smaller 3' UTRs may also reduce functional resistance rates.

Future research could explore several avenues. Related to the evolution of complex resistance, our conclusion comes with the caveats that our study examined only a single gene

drive construct, only in males, and only exclusively in European *D. melanogaster* populations. Regarding the gene drive and promoter used in this study, a less efficient gene drive or one that is not a haplolethal rescue construct may reveal greater variation across genetic backgrounds, although these inefficient constructs are less attractive for use in practice (J. Champer, Wen, et al., 2019; Du et al., 2024). Regarding the amount of genetic diversity tested here, future studies could incorporate a broader range of genetically diverse lines, such as the global diversity lines (Grenier et al., 2015), to better assess the evolution of complex resistance. However, measures of overall nucleotide diversity are only slightly higher in African and North-American populations of *D. melanogaster* compared to European populations (Kapun et al., 2021). Furthermore, a few studies have examined the impact of genetic diversity on gene drive efficiency, but none of them have reported major effects (J. Champer, Wen, et al., 2019; J. Champer et al., 2017; DiCarlo et al., 2015; Hernandes et al., 2024; Oberhofer et al., 2019; Pescod et al., 2023). J. Champer, Wen, et al. (2019) conducted a genome-wide association study (GWAS) using the Drosophila Genetic Reference Panel and reported highly heritable effects on several aspects of gene drive efficiency (particularly embryo resistance allele formation rate, and potentially cutting and homing rate). Notably, however, their analysis did not explicitly account for maternal effects or cross-level effects, not ruling out that most of that heritability was confounded by these factors (Lynch & Walsh, 1998). Interestingly, however, embryo resistance rate effects appeared consistent in some lines when using a different gene drive (J. Champer, Wen, et al., 2019). Embryo resistance rates also tended to have intermediate values (between zero and one as this is a binomial distribution), potentially allowing for genetic variation to induce larger absolute effect sizes on this performance aspect. Taking together all this evidence, we think that future studies should assess a range of gene drive constructs and effects, in both males and females, rather than testing more diverse populations of *D. melanogaster*.

Unrelated to the evolution of resistance, we think that our data points us in a direction for improving homing gene drives. Gene drive activity is usually assumed to occur independently for each offspring during meiosis, when homologous chromosomes are paired (J. Champer et al., 2017). In our hierarchical experiment, however, a large amount of variance could be attributed to sibling batch and cross-level effects. Therefore, we propose that there are developmental processes relevant for gene drive efficiency that require consideration. This hypothesis aligns with previous indications that gene drives may already be active in pre-meiotic germline stages, potentially skewing inheritance rates in either direction which

results in this kind of variation (J. Champer et al., 2017, 2018; Hammond, Karlsson, et al., 2021; López Del Amo, Bishop, et al., 2020; Verkuijl et al., 2022). Limiting the timing of a gene drive to the most optimal window for homology-directed repair, while considering the entire developmental cycle of the species, could probably yield efficiency improvements as well as reduce the amount of variance in efficiency, potentially making gene drive spread more predictable in the field. Regardless of the underlying cause, future gene drive studies should consider correcting for sibling batch (maternal and environmental effects) and cross-level effects in their experimental designs, or investigating them explicitly to better understand their potential impact on drive efficiency.

Overall, this study highlights that homing gene drives are likely still their own worst enemy, as mutations and partial homing events potentially indicative of functional simple resistance were found even in a highly conserved target gene. Arguably, this is good from a safety point-of-view, because a gene drive will not have enough power to reach fixation in non-target populations. However, a gene drive needs to be at least stable enough to be effectual to a considerable extent, otherwise there is no value in releasing it. Thus, it would still spread in non-target populations, only not to fixation. Currently, for many suppression gene drive constructs, simple resistance can curtail even the elimination of small cage populations (Carrami et al., 2018; J. Champer et al., 2017; Hammond et al., 2017; Pham et al., 2019), though in a larger, spacially structured population, it is unknown how these dynamics will behave (Dhole et al., 2020). For modification drives, simple resistance alleles are less limiting as they would usually have a much smaller selective advantage over the gene drive (Adolfi et al., 2020; Carballar-Lejarazú et al., 2023). In any case, it may be preferable to rely on construct design rather than on the chance of resistance for safety and localisation of a gene drive (Han & Champer, 2025). In contrast with simple resistance, we demonstrate that complex resistance is unlikely to be a challenge for this specific gene drive, at least in males for drive efficiency. Whether this finding extends to other scenarios remains to be determined. As there are currently some homing gene drives under consideration for field trials, we suggest that these could be opportune moments to look for complex resistance in a field setting. This screening is important, as complex resistance potentially still poses a long-term threat with large consequences for future gene drive applications in that population. Finally, we demonstrate that to test the heritability of complex resistance, an experimental design is necessary that accounts for the many potential confounding factors, the causes of which are interesting in and of itself. Our work contributes to testing

the robustness of gene drive technology in real-world settings, bringing us one step closer to harnessing their full potential for genetic biocontrol.

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Data and code availability

Data and code are available on GitHub (https://github.com/NickyFaber/HomingDrive_Heritability).

Author contributions

N.R.F., B.A.P., B.J.Z., and J.H. conceptualised the study, and all authors designed the experiments together. Experimental work, analysis, and writing were carried out by N.R.F., with support from J.C., B.A.P., B.J.Z., and J.H.. All authors read and reviewed the manuscript.

2.6 Supplementary material

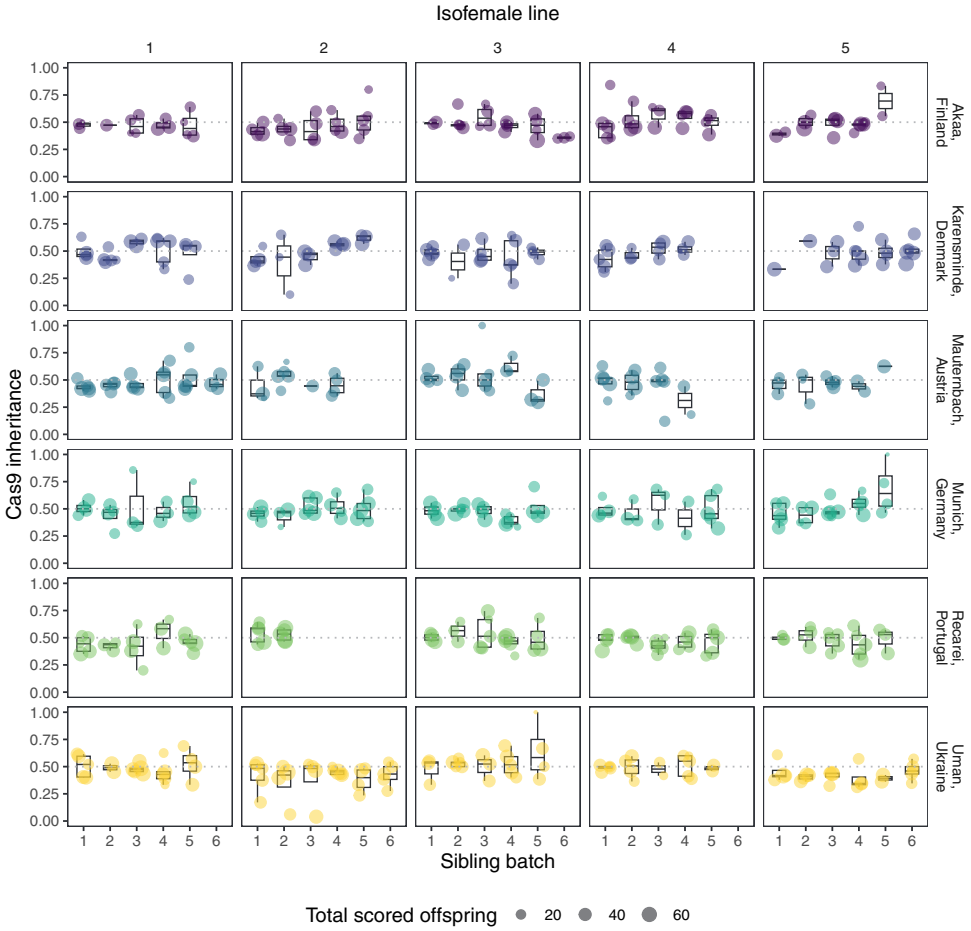


Figure S2.1. Cas9 inheritance rates plotted per each hierarchical level of the experiment. A dashed line is set at 0.5 to indicate a Mendelian inheritance rate. The size of each dot indicates the number of male offspring in that cross. In total, out of 656 crosses that were made, 579 crosses produced at least one male offspring that could be scored on fluorescence.

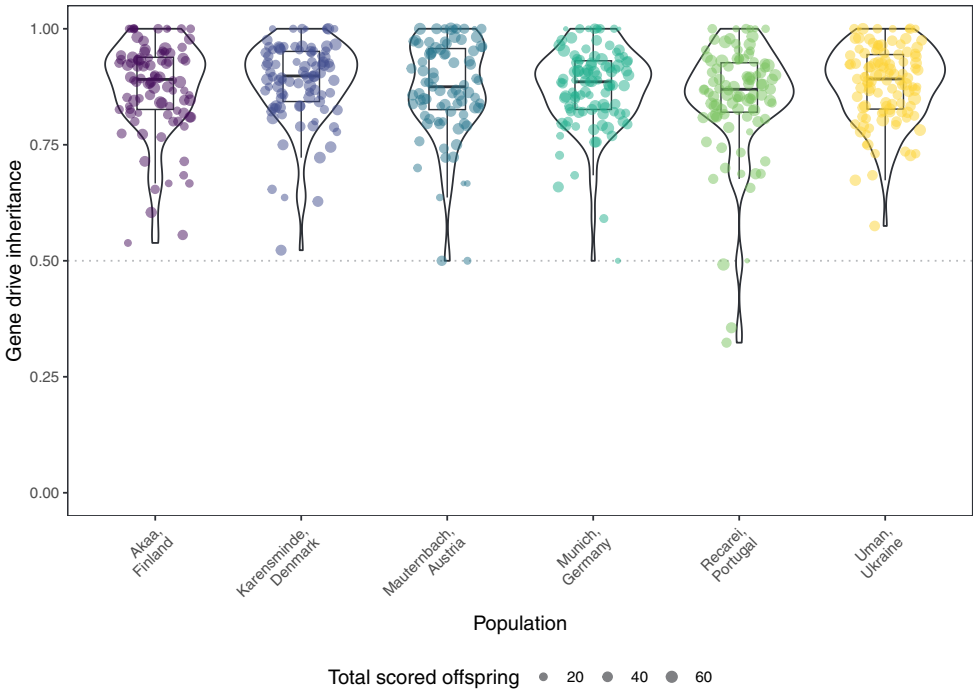


Figure S2.2. Gene drive inheritance rates plotted per population. A dashed line is set at 0.5 to indicate a Mendelian inheritance rate. The size of each dot indicates the number of male offspring in that cross. In total, out of 656 crosses that were made, 579 crosses produced at least one male offspring that could be scored on fluorescence. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. For the same plot grouped by all levels in our experiment, see Figure 2.3.

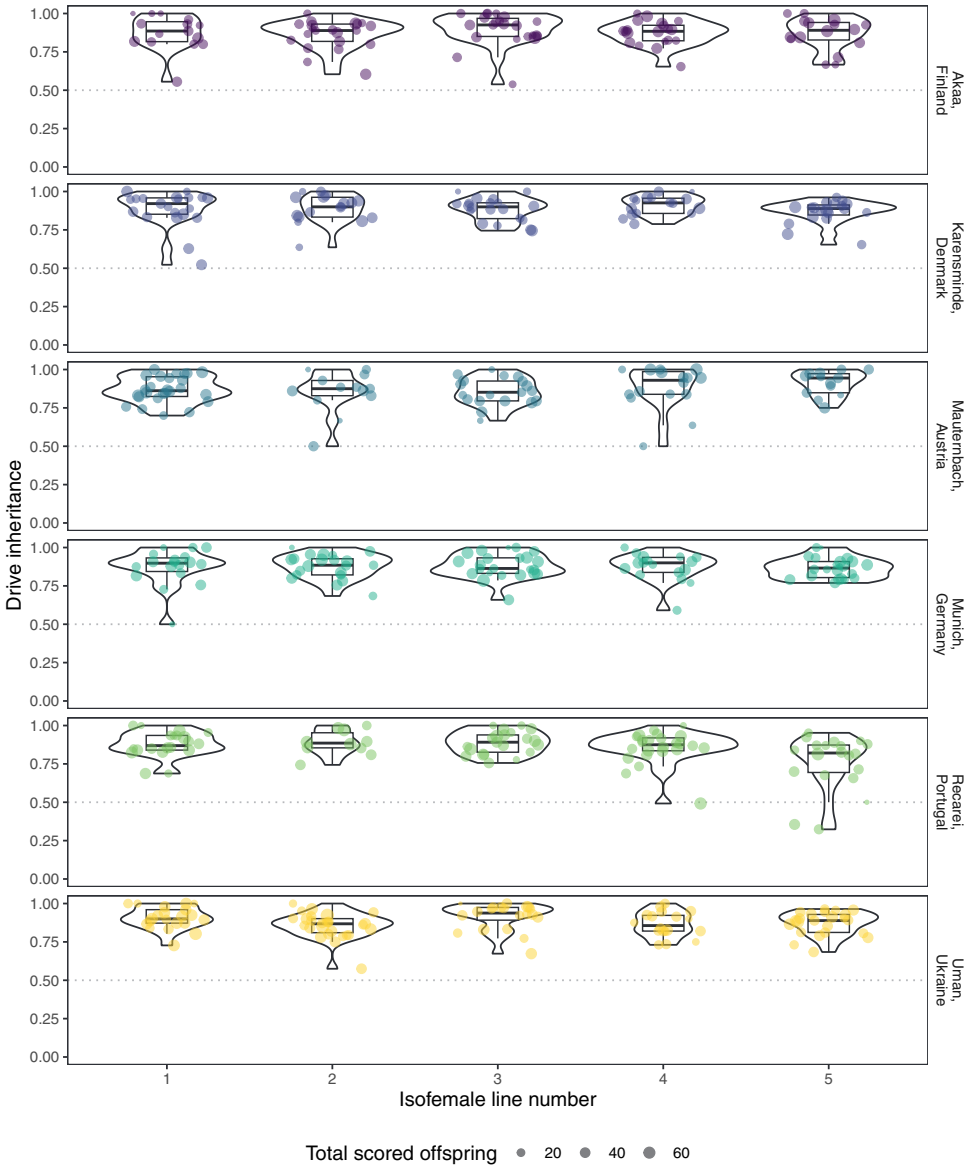


Figure S2.3. Gene drive inheritance rates plotted per population and isofemale line. A dashed line is set at 0.5 to indicate a Mendelian inheritance rate. The size of each dot indicates the number of male offspring in that cross. In total, out of 656 crosses that were made, 579 crosses produced at least one male offspring that could be scored on fluorescence. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. For the same plot grouped by all levels in our experiment, see Figure 2.3.

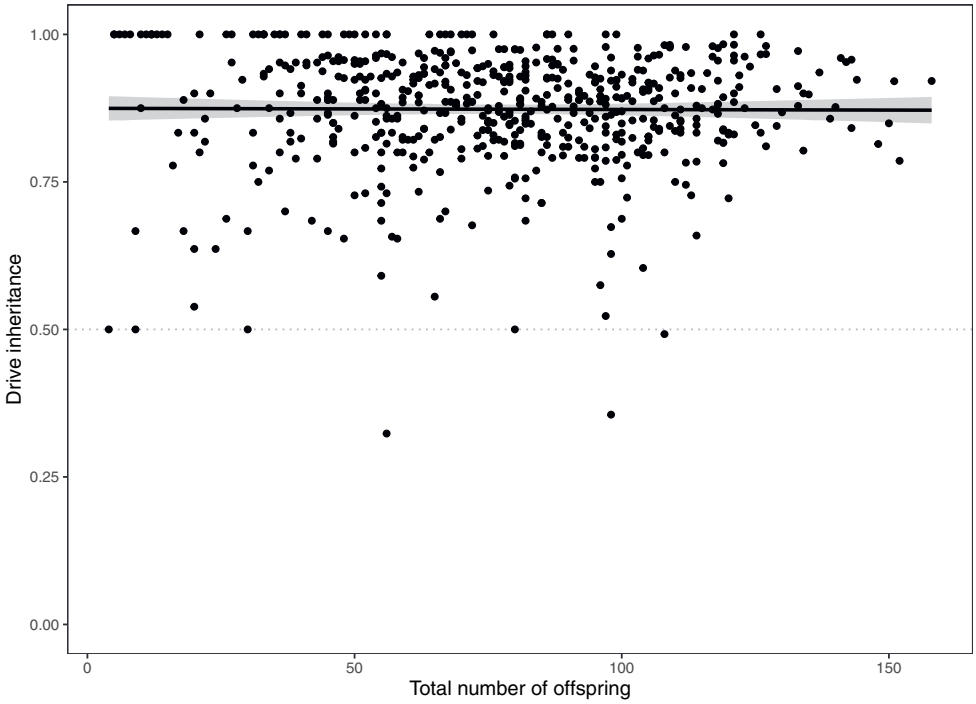


Figure S2.4. Relationship between gene drive inheritance rate and total number of offspring. Total number of offspring include all male and female offspring, as well as flies that were dead upon collection and were unable to be scored for fluorescence. The line shows the linear regression (using "lm" with formula 'y x') and the shaded area shows the 95% confidence interval.

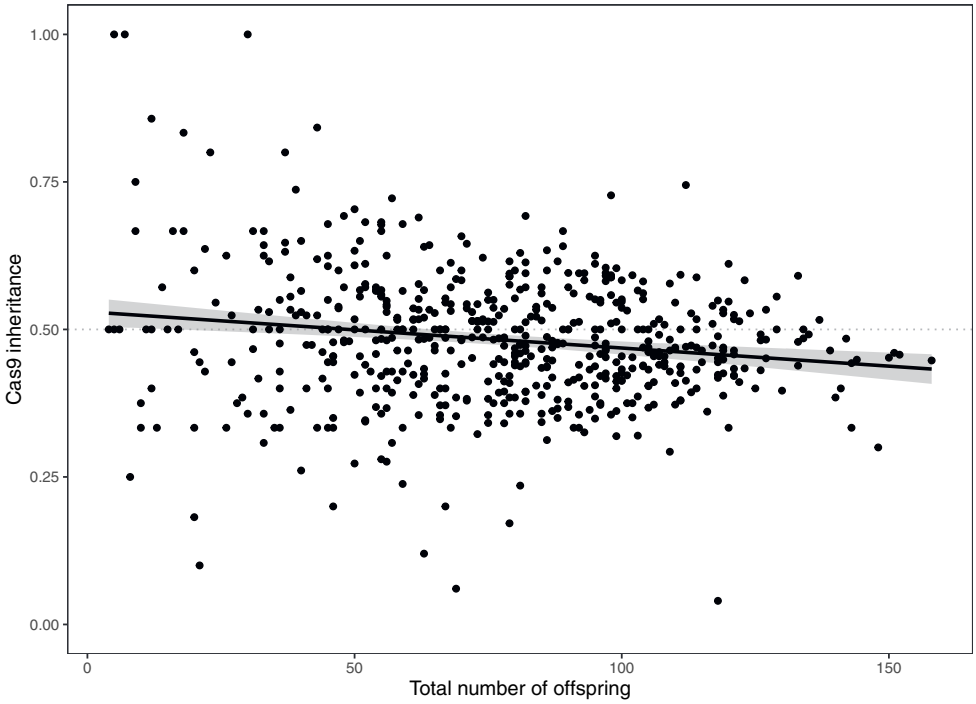


Figure S2.5. Relationship between Cas9 inheritance rate and total number of offspring. Total number of offspring include all male and female offspring, as well as flies that were dead upon collection and were unable to be scored for fluorescence. The line shows the linear regression (using "lm" with formula 'y x') and the shaded area shows the 95% confidence interval.

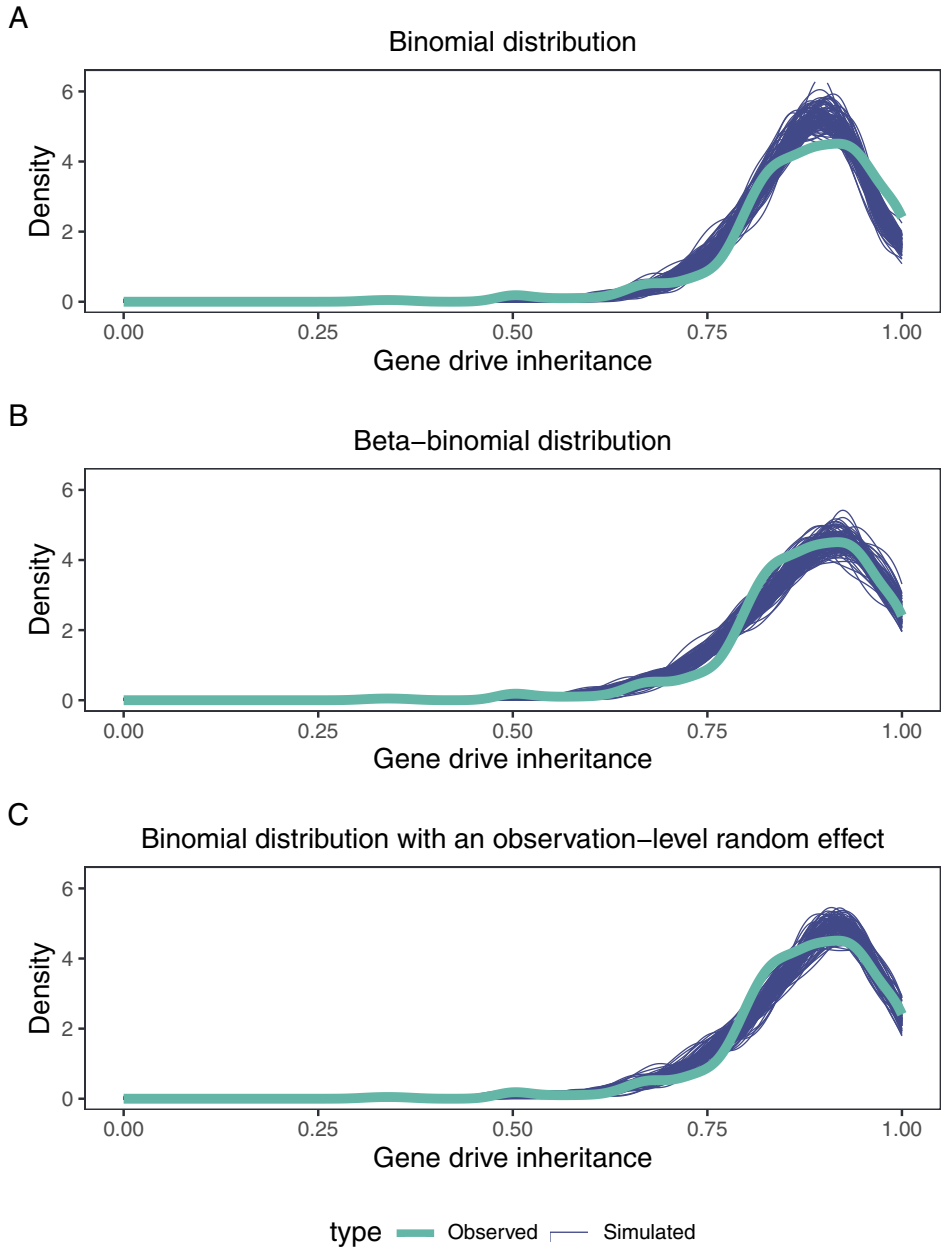


Figure S2.6. The distribution of gene drive inheritance rates in our data and those from 100 simulations of varying distributions. Simulations were done from a GLMM containing the full hierarchical model with different assumed distributions: A) binomial, B) beta-binomial, and C) binomial with an observation-level random effect.

Table S2.1. Model selection for gene drive inheritance. "OLR" stands for 'Observation-Level Random effect'.

Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
OLR_cross	2	2767.8	2776.5	-1381.9	2763.8			
OLR_batch_cross	3	2767.5	2780.6	-1380.8	2761.5	2.2340	1	0.1350
OLR_line_batch_cross	4	2768.6	2786.0	-1380.3	2760.6	0.9469	1	0.3305
OLR_population_line_batch_cross	5	2770.6	2792.4	-1380.3	2760.6	0.0000	1	1.0000
OLR_full	8	2773.1	2808.0	-1378.5	2757.1	3.4997	3	0.3208

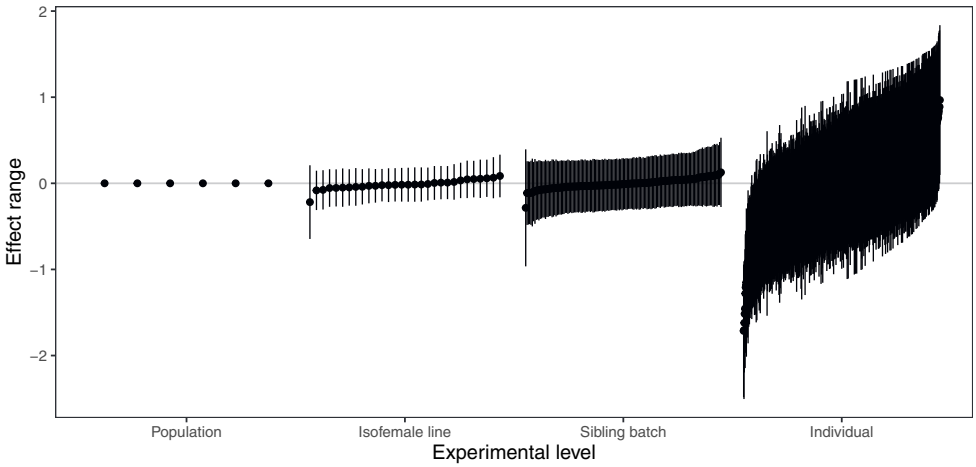


Figure S2.7. Effect ranges in each group per experimental level. Dots show the estimate of the effect of that group on gene drive inheritance (in log-link) and line ranges show the 95% confidence intervals.

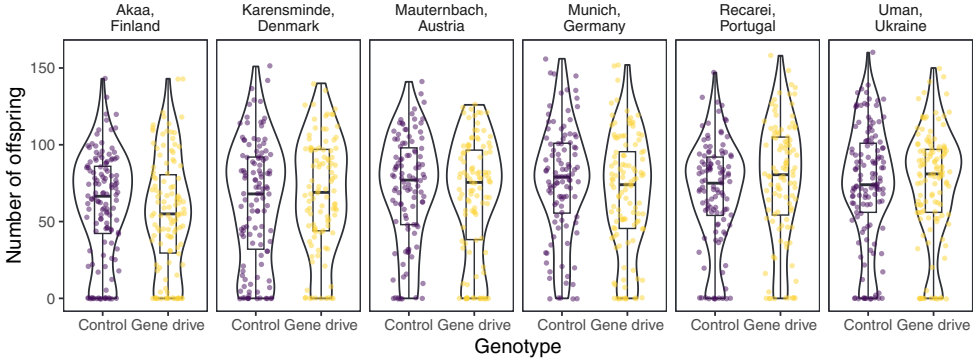


Figure S2.8. Number of offspring from gene drive and control crosses per population. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. The data is plotted per genotype in Figure 2.5.

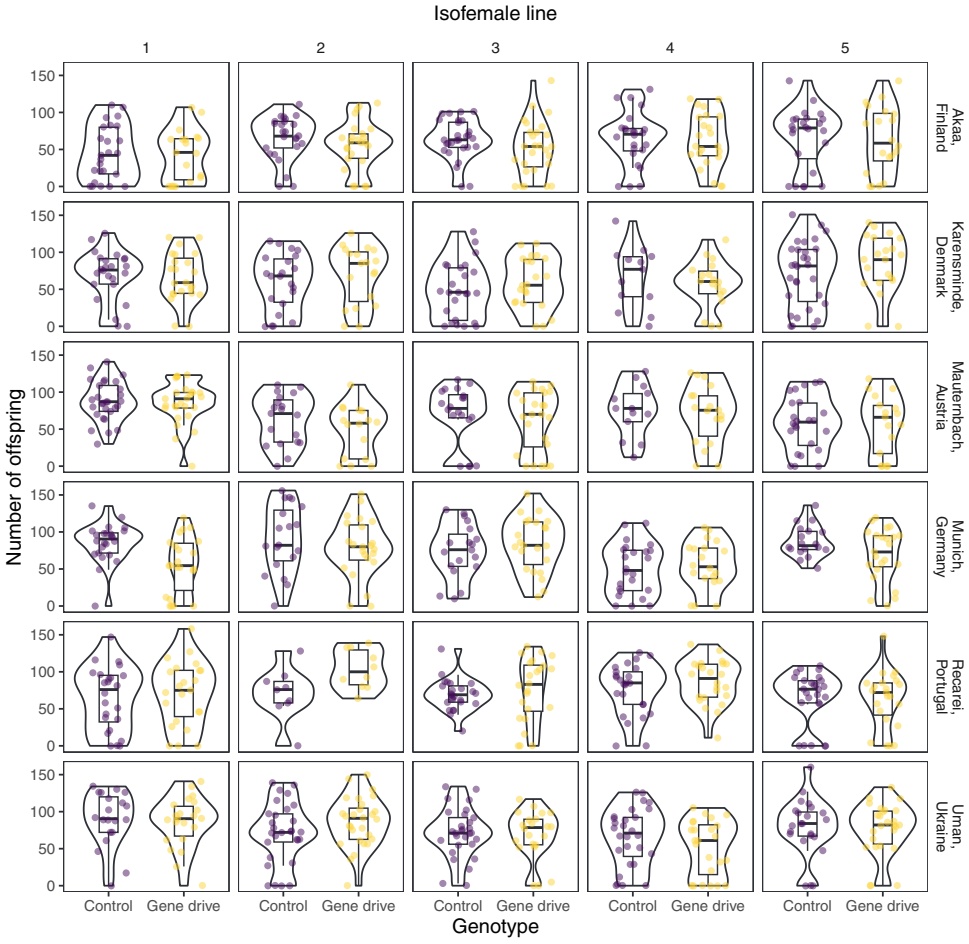


Figure S2.9. Number of offspring from gene drive and control crosses per population and isofemale line. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. The data is plotted per genotype in Figure 2.5.

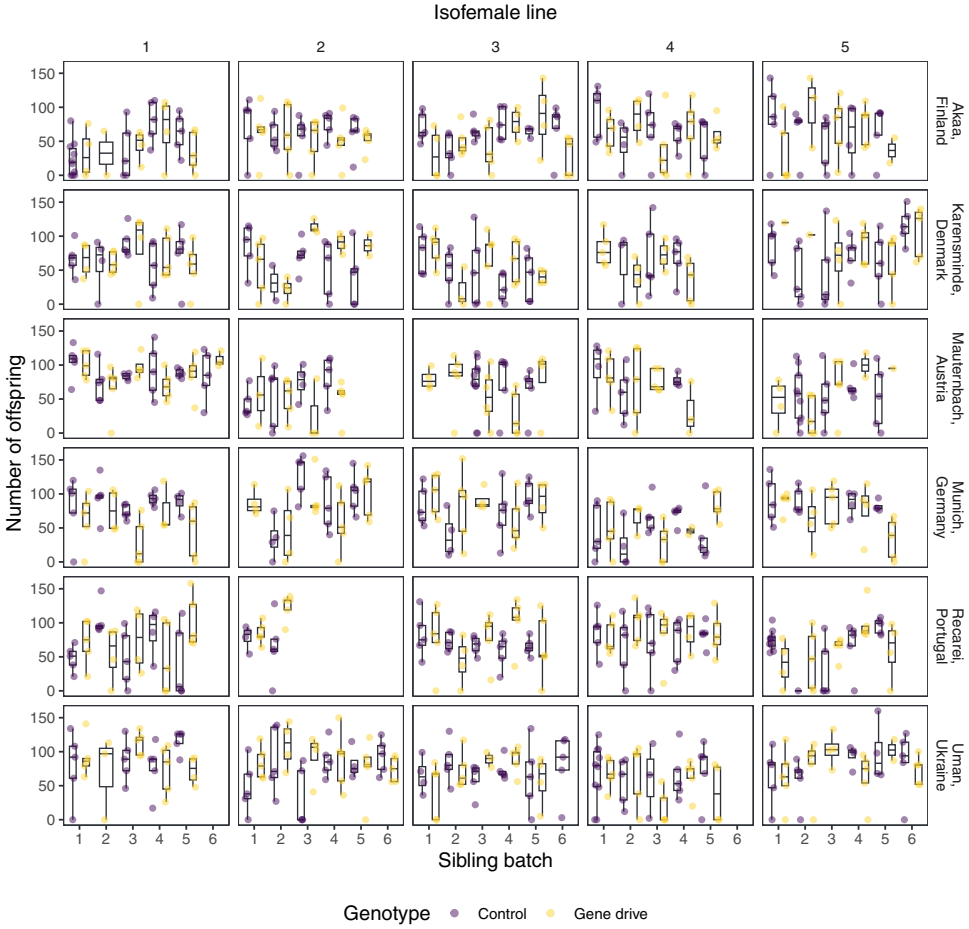


Figure S2.10. Number of offspring from gene drive and control crosses per population, isofemale line, and sibling batch. Violin plots are drawn with default "nrdo" smoothing and are proportionally sized to the number of replicates. The data is plotted per genotype in Figure 2.5.

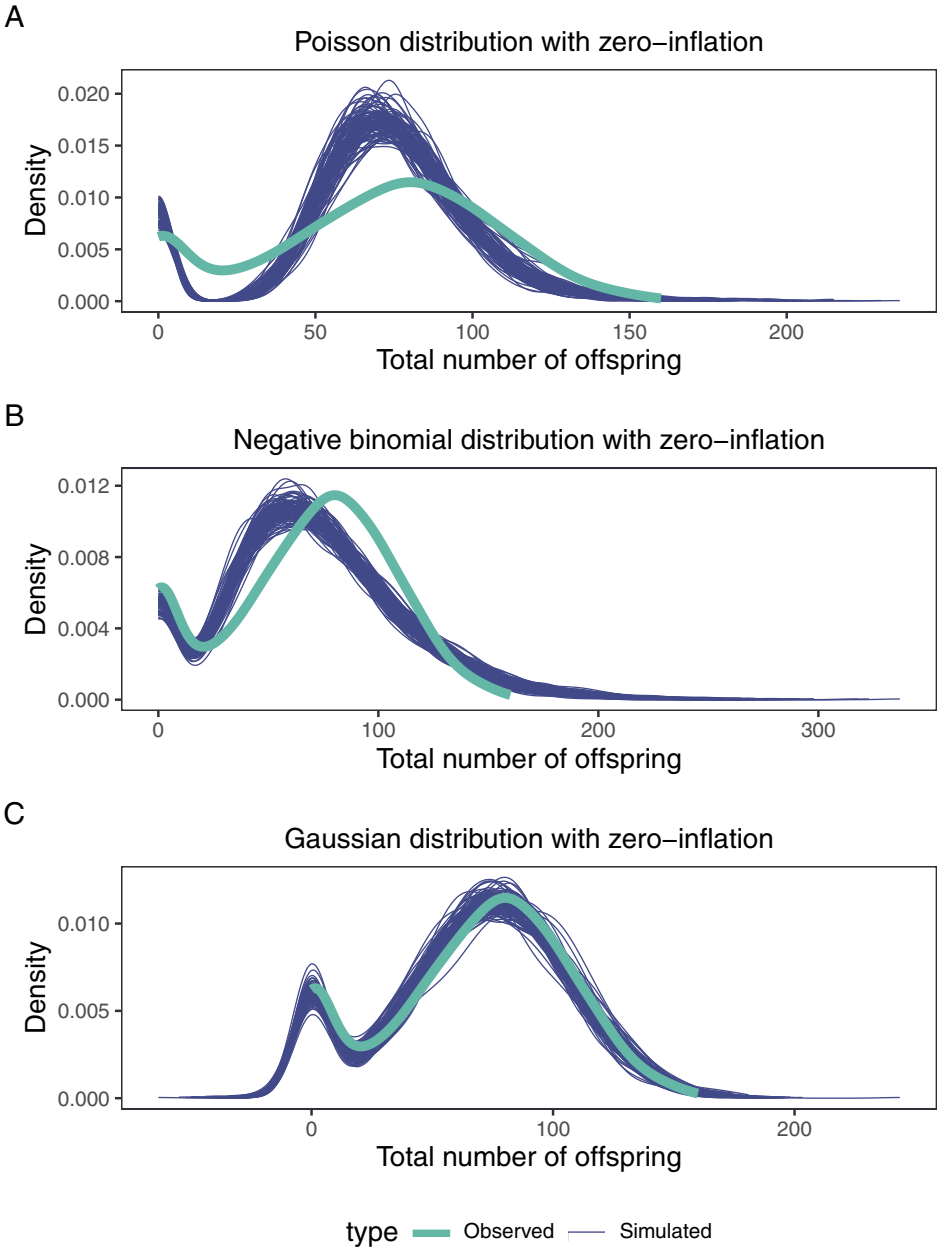


Figure S2.11. The distribution of the number of offspring in our data and those from 100 simulations of varying distributions. Simulations were done from a GLMM with a correction for zero-inflated data, and contains the full hierarchical model with different assumed distributions: A) Poisson, B) negative-binomial, and C) Gaussian.

Table S2.2. Model selection for total number of offspring. "G" stands for 'Gaussian'. A dot between two terms means it includes that interaction in the random effect, whereas two concatenated terms means only an additive effect between the two terms in the random effect.

Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)	
G_null	3	12569	12585	-6281.6	12563				
G_line	4	12526	12546	-6258.8	12518	45.5994	1	1.451e-11	***
G_population_line	5	12527	12553	-6258.5	12517	0.5691	1	0.45062	
G_population_linegenotype_mother	7	12525	12562	-6255.6	12511	5.7872	2	0.05538	.
G_populationgenotype_linegenotype_mother	8	12527	12569	-6255.6	12511	0.0000	1	1.00000	
G_populationgenotype_line.genotype_mother	9	12529	12576	-6255.6	12511	0.0000	1	0.99941	
G_population.genotype_line.genotype_mother	10	12531	12583	-6255.6	12511	0.0000	1	1.00000	
G_genotype_population.genotype ... ... _line.genotype_mother	11	12533	12590	-6255.6	12511	0.0215	1	0.88345	

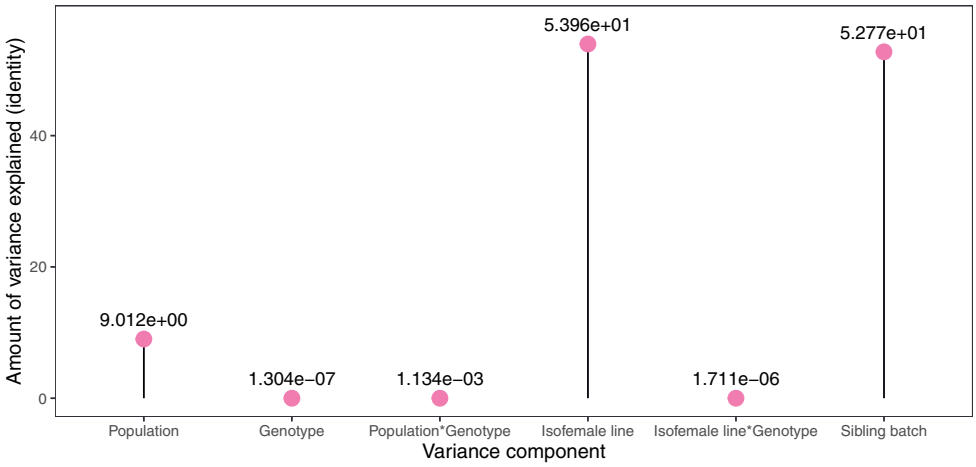


Figure S2.12. Variance in number of offspring partitioned to each hierarchical level of our experiment. These values are identity-linked.

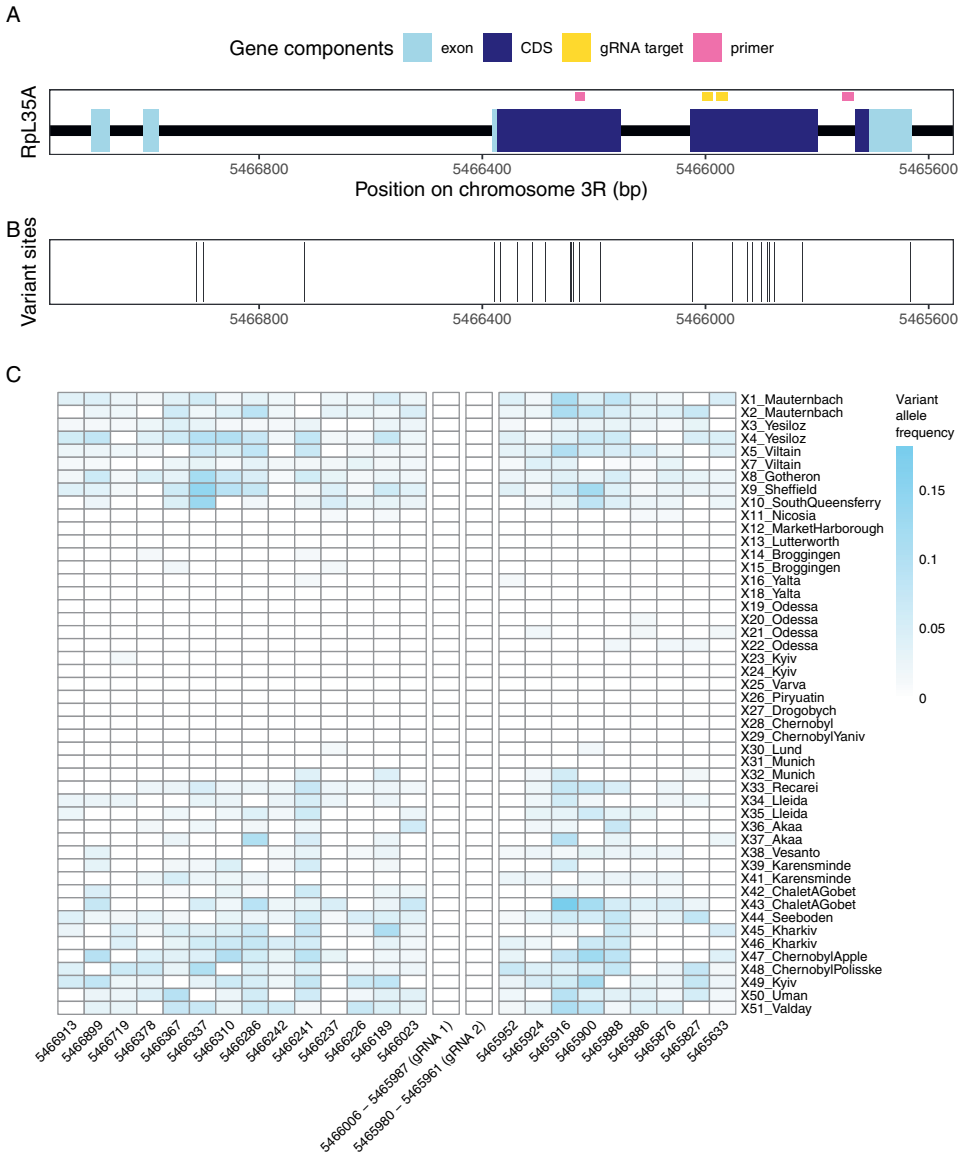


Figure S2.13. Variant sites at the *RpL35A* locus in the populations sampled by the DrosEU consortium. A) The *RpL35A* gene and its components, as well as the gene drive's two gRNA target sequences and the two primers we used for amplification. B) Variant sites in the *RpL35A* gene. C) Allele frequencies of variant sites in the *RpL35A* gene in every population sampled by the DrosEU consortium (Kapun et al., 2020), as well as the two gRNA target sequences (where there are no variants).

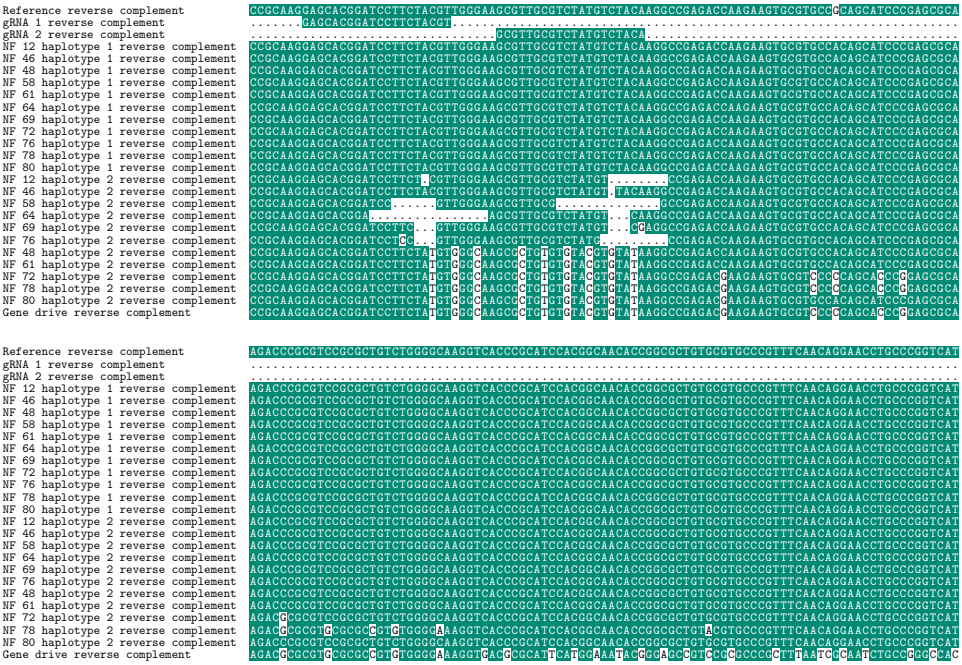


Figure S2.14. Multiple sequence alignment of the 11 observed RpL35A mutations. On top is the *D. melanogaster* reference genome, below which we show the gene drive's two gRNA targets. Below the reference sequence, we show first the 11 wildtype haplotypes of each sample, followed by the 11 resistance haplotypes that were found, with white blocks indicating deletions and mismatches with the reference. At the bottom, we show the gene drive sequence. The order of the second haplotype sequences (the resistance alleles) is kept the same as in Figure 2.6. For the full alignment, see the GitHub repository (https://github.com/NickyFaber/HomingDrive_Heritability).

"So many crucial events take place behind people's backs, when they aren't in a position to watch: birth and death, for instance. And the temporary oblivion of sex.
"Don't even think about it," he tells himself."

— Margaret Atwood, Oryx and Crake

"With few claims on her time and none on her soul, she turns back outside, into the woods, the green negation of all careers. [...] She reads Thoreau over wood fires at night. Shall I not have intelligence with the earth? Am I not partly leaves and vegetable mould myself? *And:* What is this Titan that has possession of me? Talk of mysteries!—Think of our life in nature,—daily to be shown matter, to come in contact with it,—rocks, trees, wind on our cheeks! the *solid* earth! the *actual* world! the *common sense*! *Contact! Contact! Who are we? Where are we?"*

— Richard Powers, The Overstory

Chapter 3

Gene drive performance depends on environmentally influenced fitness factors

This chapter is based on:

Faber, N. R., Maas, R., Tiemens, T., Boot, B., Kuijpers, R., Gouin, M., Feng, R., Champer, J., van den Heuvel, J., Pannebakker, B. A., & Zwaan, B. J. (2025). Gene drive performance depends on environmentally influenced fitness factors. *In preparation.*

3.1 Abstract

CRISPR-based homing gene drives could be powerful tools for controlling disease vectors and invasive species. Evaluations of gene drive constructs rely on cage trials, which despite efforts to perform these at large scales, are conducted in highly uniform laboratory environments. However, these necessarily lack ecological pressures such as suboptimal food quality, dispersal, predation, or weather variability. Because fitness is strongly context-dependent, drive performance observed in the laboratory may not translate readily to the field. Here, we take a first step towards assessing how environmental variation may influence homing gene drive efficiency. We focus on a single environmental factor relevant for *Drosophila melanogaster*: the yeast availability in the larval and adult fly medium. Yeast supplies nearly all nutrients beyond sugar, and affects life-history traits in non-linear and life stage–specific ways, including reproduction, lifespan, and choice of DNA repair pathway. We tested a haplolethal modification gene drive in a population setting and find significant differences in how the gene drive spreads across yeast environments. Because the gene drive inheritance rate itself remained stable, we conclude that these differences must come from variable fitness effects. Fitness experiments implicated variation in egg-laying rate, egg and larval survival, and perhaps also development time and female survival. Our results indicate that even simple environmental shifts can alter gene drive performance, underscoring the need to account for the ecological context before gene drive field trials are considered or attempted.

3.2 Introduction

CRISPR-based gene drives could be a valuable tool for managing vectors of disease or invasive pests to ultimately improve human and ecological health (Esvelt et al., 2014). Gene drives are genetic constructs, designed to spread through a population over generations. They either carry fitness-neutral effects, such as immunising the malaria mosquito against the malaria parasite (Carballar-Lejarazú et al., 2020), or detrimental effects to the carrier, for example inducing female sterility for the goal of population suppression (Kyrou et al., 2018). Homing gene drives, the most powerful type of gene drive, can increase in frequency after releasing only a few gene drive individuals in a population (Hammond, Pollegioni, et al., 2021). These gene drives spread by replacing their counterpart wild-type allele in heterozygotes, thus achieving inheritance rates higher than the usual 50%, and ideally approaching 100%. The gene drive copies itself onto the homologous chromosome by expressing Cas9 and a guide RNA (gRNA) that target the homologous wild-type locus at the insertion site of the gene drive. After a double-stranded break is induced, homology-directed repair uses the drive-bearing chromosome as repair template, effectively copying the gene drive. Homing gene drives have been tested in a range of species including yeast (Shapiro et al., 2018), fruit flies (J. Champer et al., 2017; Gantz et al., 2015; Yadav et al., 2023), several genera of mosquitoes (Carballar-Lejarazú et al., 2020; Feng et al., 2021; Hammond et al., 2016; Kyrou et al., 2018; M. Li et al., 2020), the diamond-back moth (X. Xu et al., 2022), mice (Grunwald et al., 2019; Pfitzner et al., 2020), and rats (C. Lai et al., 2022). In the malaria mosquito *Anopheles gambiae*, achieved gene drive efficiencies are so high that field trials are now under consideration (Connolly et al., 2024; Long et al., 2020).

In general, homing gene drive efficiency depends on two key factors: the gene drive inheritance rate and gene drive carrier fitness (Deredec et al., 2008). Gene drives with higher inheritance rates, from the standard 50% towards 100%, can spread more rapidly through a population. However, this increased inheritance can be (partially) off-set if gene drive carriers experience a fitness costs. Many molecular factors can impact drive carrier fitness. First, somatic expression of drive components (as opposed to the generally desirable germline-only expression) can lead to reduced fitness (E. Yang et al., 2022). Second, maternal deposition of drive components into oocytes can lead to premature cleavage of the paternal genotype in somatic cells of the developing embryo, leading to somatic mosaicism and fitness costs (Carballar-Lejarazú et al., 2022; J. Champer et al., 2017; López Del Amo, Bishop,

et al., 2020). Third, some modification gene drives contain recoded rescue sequences for the gene they are inserted into (J. Champer, Yang, et al., 2020; Kandul et al., 2021), which may provide only incomplete or imperfect rescue. Fourth, off-target Cas9 cutting has been hypothesised to induce fitness effects, though no direct evidence of this was found (Langmüller et al., 2022). Fifth, potential impacts of other drive elements may include the mere presence of fluorescence proteins and Cas9 proteins in the cytosol, although this effect has been shown to be unlikely (Langmüller et al., 2022). Finally, in some drive systems, inheritance and reproductive output are linked because failed drive activity can generate lethal genotypes (J. Champer, Yang, et al., 2020). Because so many factors can impact the fitness of drive carriers, it is impractical to quantify all of them for every gene drive construct. Therefore, most studies measure inheritance rate and reproductive success only in single crosses before proceeding to cage trials to assess drive efficiency.

Cage trials are a sensible first step for testing gene drive systems in populations towards their eventual release in the field. However, they necessarily simplify natural environments. Even large cage trials occur in highly uniform laboratory environments: nutrient-rich food, stable temperature and humidity, somewhat controlled larval density, no weather events, no dispersal opportunities, no predators, and none of the other ecological pressures present in natural settings (Hammond, Pollegioni, et al., 2021). However, fitness is strongly environment-dependent and always relative to other individuals of the same population or those of surrounding populations (Fisher, 1999). Once gene drive carriers are released into the wild, it is inevitable that they face harsher, more variable circumstances compared to the laboratory. Should this environmental variability impact gene drive individuals more significantly than wild-type individuals, this could lead to more variable drive performance or unexpected failure to spread. Thus, the ecology of a population is likely a highly relevant determinant of drive performance. So far, no gene drive system has been tested in the field. The only preliminary test conducted in mosquitoes released a non-CRISPR, inactive construct to assess carrier fitness (Yao et al., 2022). Although no fitness costs were detected in the laboratory assays, carriers showed significant fitness effects in the field through reduced survival and migration rates.

Laboratory adaptation is a well-known phenomenon that causes populations reared in captivity for some generations to lose some of their stress resilience (Hoffmann et al., 2001). For genetic biocontrol technologies like sterile insect technique (SIT), where insects are

mass-reared in laboratories and released at high numbers and frequent intervals, this factor significantly contributes to reduced fitness in the field (Bouyer & Vreysen, 2019; Dyck et al., 2021). For a gene drive, however, this factor may be less relevant: due to the way a homing gene drive copies itself, it will quickly introgress into the genetic background of the wild population (Tushar et al., 2024), and likely benefit from any genetic adaptations that the wild populations has. Therefore, the most important fitness costs will likely not be related to lab rearing and release strategies, but to the gene drive itself.

We have taken a first step towards assessing how environmental variability may influence the efficiency of a homing gene drive. Specifically, we tested the influence of a single environmental factor on gene drive performance in *Drosophila melanogaster*: the concentration of yeast in the larval and adult medium. In *D. melanogaster* medium, sugar provides the carbon source and yeast supplies nearly all remaining essential nutrients including proteins, fats, vitamins, electrolytes, and other micronutrients (Ashburner, 1989). As a result, most life-history traits related to fitness depend on the amount of yeast available, including body size, reproduction, developmental speed, health, and lifespan (Anagnostou et al., 2010). Importantly, the influence of yeast is both non-linear and life stage-specific (Jang & Lee, 2018; May & Zwaan, 2017). For example, larvae reared on low-yeast diets develop into adults that are more fecund than those from higher-yeast conditions (May & Zwaan, 2017). Additionally, the choice of DNA repair pathway, either end joining (EJ) or homology directed repair (HDR), has also been shown to partially depend on the reproductive activity and health of flies, and only the latter repair pathway leads to successful homing of the drive drive (A. D. Wang & Agrawal, 2012). Taken together, these findings suggest that gene drive efficiency could be affected by yeast availability, either through changes in DNA repair pathways and/or through fitness effects that are currently not routinely measured.

In this study, we focused on a haplolethal modification gene drive (J. Champer, Yang, et al., 2020) in combination with the *CG4415* promoter and a *nanos* 3'UTR for Cas9, which was a combination shown to lead to minimal fitness costs due to embryo deposition (Du et al., 2024). However, this promoter was also shown to induce somewhat more somatic drive expression than the standard *nanos* promoter, which could lead to fitness costs when combined with a haplolethal gene drive. First, we observed significant effects of yeast concentration on drive performance in population trials. Second, we began to unravel the poten-

tial causal factors behind these effects. Because drive inheritance rates remained consistent across treatments, we concluded that the variation must arise from differences related to fitness components. We found that there is not a single dominant fitness aspect, but many fitness effects act simultaneously: egg-laying rate of drive females is implicated, as well as egg and/or larval survival, and perhaps also female survival and development time. Some factors, including mate choice and male fertility, had no measurable impact on drive carrier fitness. In a second set of population trials, we found that this gene drive is threshold-dependent, likely due to heterozygote disadvantage. Together, these findings indicate that environmental variation exerts diffuse, but cumulatively relevant influences on overall gene drive carrier fitness and resulting efficiency. Our results warrant for caution starting gene drive trials in natural population without detailed knowledge on gene drive fitness effects and their ecological dependencies.

3.3 Results

3.3.1 Cage populations with variable amounts of yeast in the medium

In our first experiment, we varied the amount of yeast in the fly medium (to either half (low) or twice as much (high) yeast compared to the standard (intermediate) concentration) and measured how the gene drive performed (Figure 3.1A). Populations were started with 48 flies, 16 of which were heterozygous for the gene drive (but without Cas9 in the case of the controls). Thus, the gene drive started at a carrier frequency of 33%.

We observed that the gene drive was not able to spread in any replicate, but that in fact, the gene drive frequency decreased in populations with an active gene drive compared to the inactive drive control. The gene drive in the control populations (carrying the gene drive but not Cas9) consistently lingered at the initial frequency. Combined, these results indicate that the decrease in gene drive frequency is due to Cas9 activity, and not due to the gene drive construct itself. Interestingly, the frequency of the gene drive does not seem to decrease at an equal rate across the different yeast concentrations, indicating that the nutritional environment impacted drive efficiency. In a high yeast environment, the construct lingers roughly around the initial frequency, similar to the control populations, as opposed to the quick decline of the gene drive in low and intermediate yeast environments.

These effects are shown to be statistically significant by fitting a linear model to the gene

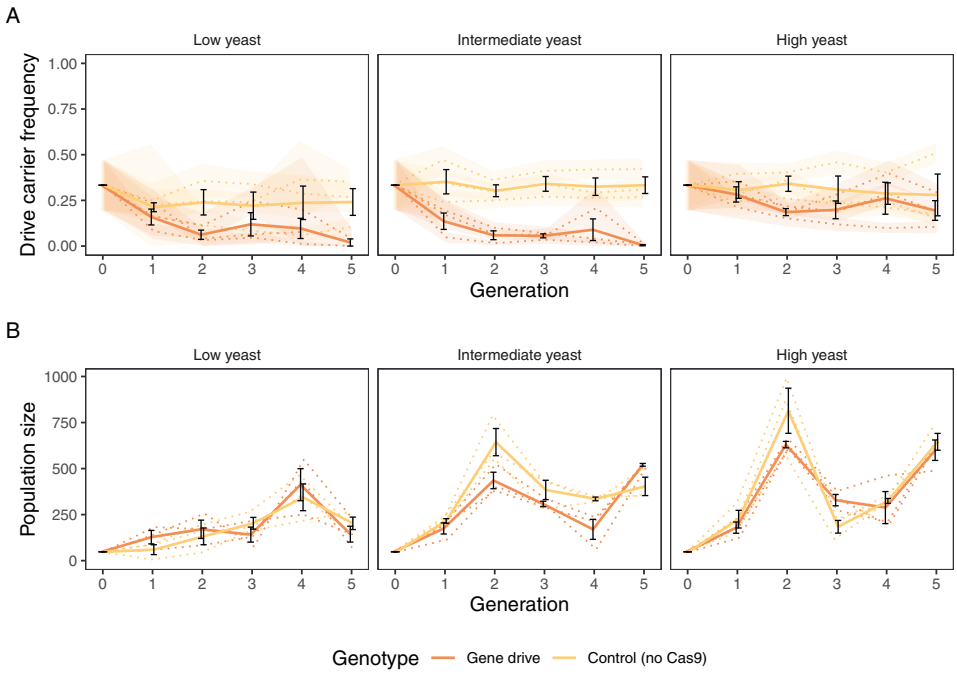


Figure 3.1. Cage study of the gene drive in populations on different yeast concentrations. A) Gene drive carrier frequency over time, where the dotted lines represent the three individual replicates and the translucent ribbons indicate the standard error around the mean in each replicate (depending on the number of flies scored for fluorescence). The solid line and error bars represent the mean and standard error around the mean over the three replicates together. B) Population size over time, where the dotted lines represent the three individual replicates, and solid lines and error bars represent the mean and standard error around the mean.

drive frequency with an autocorrelation effect stretching one generation. We found significant effects of genotype (gene drive or control) and generation, as well as an interaction between genotype and generation and an interaction between yeast concentration, genotype, and generation (see Table S3.1 for model selection and and Table S3.2 for the significant effects in the best-fitting model). The former interaction indicates that drive and control genotypes showed differing dynamics over time, while the latter shows that drive and/or

control genotypes did not act the same over time in different yeast environments. We conclude that a high yeast environment must either increase drive inheritance rate or somehow alleviate some of the fitness costs that prevented the spread of this gene drive.

3.3.2 Drive inheritance

We tested gene drive inheritance in both male and female drive carriers, again either with Cas9 (gene drive) or without (control) (Figure 3.2). As mentioned in the introduction, low yeast flies are more likely to use HDR relative to EJ (A. D. Wang & Agrawal, 2012), which takes more time and is more costly as less energy is available for reproduction. Conversely, high yeast flies preferably use EJ as a repair mechanism, which leaves more energy for reproduction. In the case of our haplolethal gene drive, EJ repair will lead to a higher amount of lethal genotypes, reducing the amount of offspring.

Similar to the studies that tested our gene drive and promoter previously (J. Champer, Yang, et al., 2020; Du et al., 2024), we found that the gene drive skews its inheritance to significantly above 50% in both male and female drive carriers, as opposed to the controls, where inheritance was consistently around 50%. Gene drive inheritance was overall higher in males (91%) than in females (63%). Fitting a linear model to this data, gene drive inheritance depends significantly on genotype (drive or control), drive parent (male or female), as well as the interaction between the two (see Table S3.3 and Table S3.4 for model selection and statistical significance). Most importantly, however, we determined that the environment had no significant impact on inheritance rates in males or females, excluding the possibility that the relative use of the various DNA repair pathways differed depending on the environment in our cage experiment.

3.3.3 Estimating drive carrier fitness

For the gene drive to decrease in frequency, despite a high homing rate in males and an intermediate homing rate in females, the fitness of drive carriers must be very low. Perhaps a subtle indication of a fitness disadvantage associated with the gene drive was observed in the overall population size in the first and second generation (Figure 3.1B). In all environments, populations showed a characteristic initial exponential increase in size, and a subsequent decrease due to the carrying capacity of the bottles being reached and density and competition effects taking over (Pomerantz et al., 1980; Thomas et al., 1980). Notably, in interme-

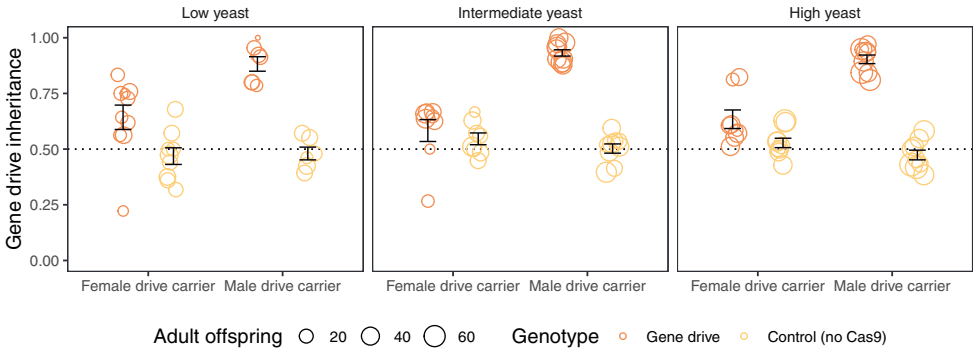


Figure 3.2. Single crosses on different yeast concentrations to determine gene drive inheritance. Gene drive inheritance was measured in both male and female drive carriers, and either containing Cas9 (gene drive) or no Cas9 (control). All drive carriers were heterozygous for the drive, and crossed to counterpart *w*^{1118} flies. Error bars represent the mean and standard error around the mean.

diate and high yeast environments, the control populations grew faster than the gene drive populations, indicating that the presence of the gene drive induced a genetic load on the population. When fitting a linear model on population size in the second generation, we found a significant effect of both yeast and genotype, as well as an interaction between the two, which likely indicates that the reduced population size of gene drive populations did not hold for a low yeast environment (see Table S3.5 and Table S3.6). This reduced population size vanished later as carrying capacity was reached, probably because the genetic load is compensated for by density-dependent effects, that is, reduced competition.

To estimate the fitness of drive carrying individuals, previous studies have used a Maximum-Likelihood Estimation model that includes high-level life history traits and can be adapted to include gene drive inheritance and activity (J. Liu et al., 2019). In this model, three fitness components can impact fitness per generation: viability, mate choice, and fecundity. The viability fitness component depends on the genotype of each individual, both females and males, and determines survival until reproduction. The mate choice component is based on female preference for males of specific genotypes and thus, only affects males. The fecundity fitness component is assumed to be a result of the female genotype. As it is unclear what proportions to implement for each of these three factors in the model, usually only viability selection is used, or a combination of mating and fecundity selection

Table 3.1. Fitness component structure in the two Maximum-Likelihood Estimation models used to estimate fitness components of the gene drive. The viability fitness model affects both males and females, while the combined mating and fecundity fitness model affects only males or females, respectively. Fitness costs are assumed to be multiplicative, so heterozygote fitness is the square root of the homozygote fitness.

	Fitness component model	
Genotype	Viability	Mating = Fecundity
Wild-type (w^{1118})	1	1 = 1
Drive heterozygote	$\sqrt{x}$	$\sqrt{y} = \sqrt{z}$
Drive homozygote	x	$y = z$

assuming equal proportions (Table 3.1). Fitness costs are assumed to be multiplicative going from heterozygotes to homozygotes. Inheritance rules for the gene drive are based on our observed inheritance rates from Figure 3.2, as well as an assumed germline resistance rate of 1/10th of the conversion rate, and no embryo deposition or functional resistance.

Following the assumptions of the viability and combined mating and fecundity models, we have estimated the fitness components of the gene drive separately in each environment (Table 3.2) based on our observed drive frequency changes (Figure 3.1). We found that mating fitness and fecundity of drive homozygotes range between 0.14 and 0.35 to account for the gene drive frequency decline. The 95% confidence intervals of these fitness estimate do not overlap between the high and intermediate yeast concentrations, although there is some overlap between high and low yeast concentrations, indicating a significant impact of the environment on gene drive fitness components.

3.3.4 Female fecundity and egg survival

To dig deeper into the cause of the differing drive carrier fitness across environments, the first life history trait we investigated was female fecundity. For the crosses we used to determine drive inheritance in Figure 3.2, we also measured egg laying and egg-to-adult survival in the three different environments.

In terms of egg laying (see Table S3.7 and Table S3.8 for model selection and statistical signif-

Table 3.2. Maximum-Likelihood Estimation of fitness components of the gene drive (J. Liu et al., 2019). All fitness components are estimated for homozygote individuals; for heterozygotes, the fitness is the square root of these values, as we assume there is no dominance (Table 3.1). In brackets is the 95% confidence interval around the fitness component estimate. For this model, we use our observed drive inheritance rates (Figure 3.2), and we assume a viability fitness of 1.0, a germline resistance rate of 1/10th of the drive conversion rate, an embryo resistance formation rate of 0.0, and a relative r1 (functional resistance allele) formation rate of 0.0 (Du et al., 2024).

Environment	Fitness cost model	Log-likelihood	AICC	Effective population size	Viability fitness	Mating fitness = Fecundity fitness
High yeast	Mating-fecundity	13.4	-21.5	30 [11 - 61]	1	0.35 [0.25 - 0.48]
Intermediate yeast	Mating-fecundity	25.1	-45.2	25 [6 - 34]	1	0.14 [0.07 - 0.23]
Low yeast	Mating-fecundity	21.4	-37.7	25 [6 - 37]	1	0.17 [0.10 - 0.28]
High yeast	Viability	11.1	-16.8	25 [6 - 42]	0.26 [0.15 - 0.41]	1
Intermediate yeast	Viability	23.6	-42.2	25 [5 - 32]	0.05 [0.02 - 0.11]	1
Low yeast	Viability	19.1	-33.1	25 [4 - 32]	0.08 [0.04 - 0.16]	1

icance), as expected, the amount of yeast in the environment is a significant determining factor (Figure 3.3A), with flies laying fewer eggs on low yeast medium and more eggs on high yeast medium. There is no significant difference in the number of eggs laid between gene drive and control flies. Interestingly, there is a significant difference in the number of eggs laid by females in crosses containing female and male carriers (as each drive carrier was paired with a w^{1118} "wild-type" partner, w^{1118} females' fecundity was measured for male drive carriers). Apparently, female drive carriers lay significantly fewer eggs than w^{1118} females. However, although batch effects were controlled by rearing all parental crosses at low densities, we cannot exclude that they contributed to the observed difference. Unfortunately, we neglected to include w^{1118}-only crosses in this experiment, which could have excluded the option that w^{1118} females lay fewer eggs due to poor quality sperm from gene drive males.

Egg-to-adult survival rate (see Table S3.9 and Table S3.10 for model selection and statistical significance) is overall significantly negatively impacted by a gene drive genotype compared to the control (Figure 3.3B), although effect sizes are relatively small. The other two factors, yeast concentration and drive parent (male or female) had no significant influence by themselves, but their interaction was significant. The interpretation of this result is unclear, as differences are small throughout.

Taken together, these results provide support for the notion that gene drive activity only mildly impacts female fecundity in different ways in different environments. Although there are some effects in terms of egg laying (Table S3.8) and egg survival (Table S3.10), a relatively small amount of variance was explained by these factors and overall differences between groups were modest. Certainly, we do not observe a fecundity fitness close to $\sqrt{0.14} = 0.37$ or $\sqrt{0.35} = 0.59$ as estimated from our cage study (Table 3.2, taking the square root of the fitness parameter estimate as heterozygotes were measured).

3.3.5 Mate choice

A second factor that could contribute to a strong fitness cost for gene drive carrying individuals could be mate selection by females against gene drive males. In this assay, we gave w^{1118} females the choice between two males: one w^{1118} male and one gene drive or control male. This assay was only conducted in bottles with intermediate yeast, as flies are not expected to meaningfully interact with the medium while competition for mating was on-

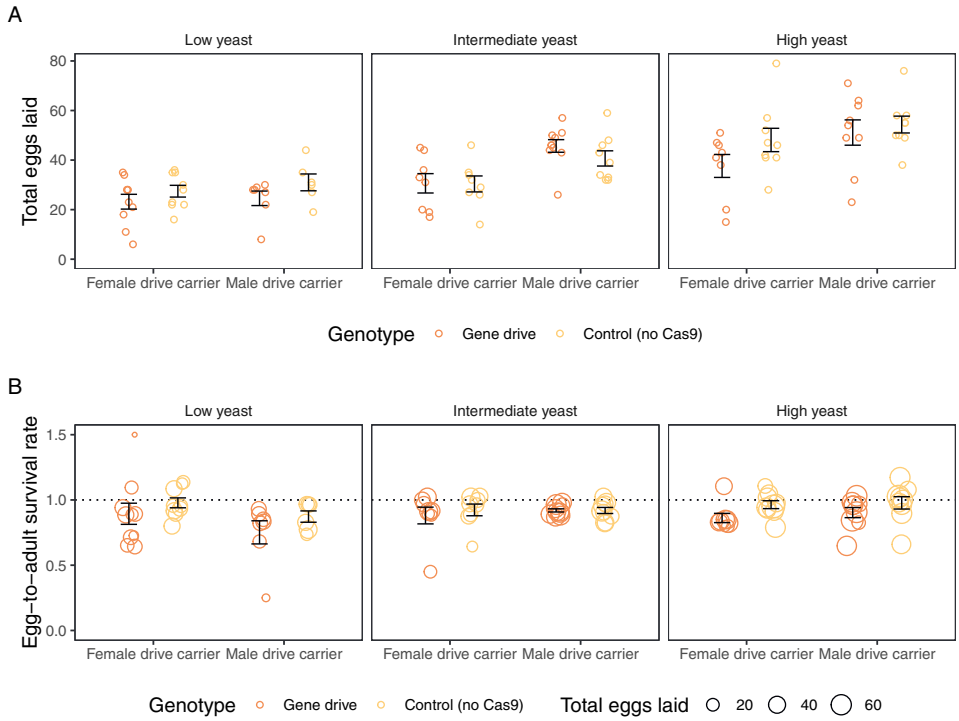


Figure 3.3. Single gene drive crosses on different yeast concentrations where egg laying (A) and egg-to-adult viability (B) were measured. All drive carriers were heterozygous for the drive. Note that egg survival is sometimes slightly over 100% due to eggs that were missed during counting. Error bars represent the mean and standard error around the mean.

going. Therefore, this fitness cost is unlikely to explain the difference between gene drive performance across yeast concentrations.

We observed that females chose gene drive males over w^{1118} males at a 0.58 rate, and that females chose control males over w^{1118} males at a 0.46 rate (Figure 3.4). Neither of these rates is significantly higher or lower than the expected 0.5, indicating no preference or avoidance of genetically modified flies (Exact binomial test two-sided, for gene drive males N = 50 and p = 0.32, and for control males N = 48 and p = 0.67). We conclude that mating fitness is likely not a factor explaining the failure of the gene drive in our population experiments either. Similar to female fecundity, mating fitness as estimated from our population study

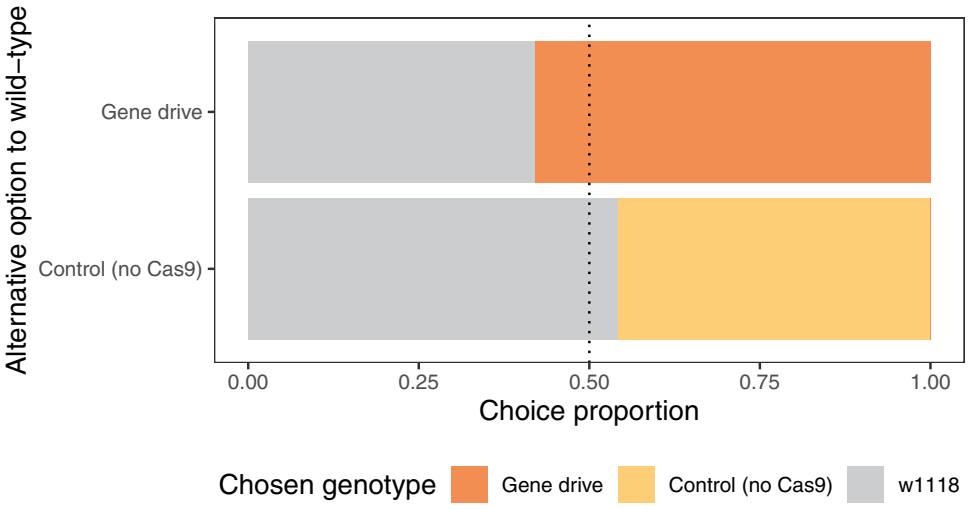


Figure 3.4. Female mate choice assay. All drive carriers and controls were heterozygous for the drive. This assay was performed in an intermediate yeast environment only.

does not line up with these findings ($\sqrt{0.14} = 0.37$ or $\sqrt{0.35} = 0.59$, Table 3.2).

3.3.6 Male fertility

Next, we tested the fertility of males, a factor often neglected in evolutionary studies as males are usually not the limiting factor for a population's reproductive output. However, in the case of males carrying a gene drive that could induce lethal genotypes, male fertility might be a highly relevant factor. We gave a single male (of either gene drive, control, or w^{1118} genotype) seven virgin w^{1118} females and allowed them to mate for 24 hours. Subsequently, the male was removed and females were transferred to individual vials and the number of offspring produced over five days of egg laying was counted.

Although gene drive males had a seemingly reduced fertility on intermediate yeast, this effect is not consistent in the other environments and variance is high (Figure 3.5). We also did not observe clear differences in how many out of the seven females were inseminated between groups (Figure S3.1). Most females were inseminated, and variance in the number of offspring per females seemed equal as well. When fitting a linear model to the total number of offspring produced per male, only yeast concentration had a significant effect

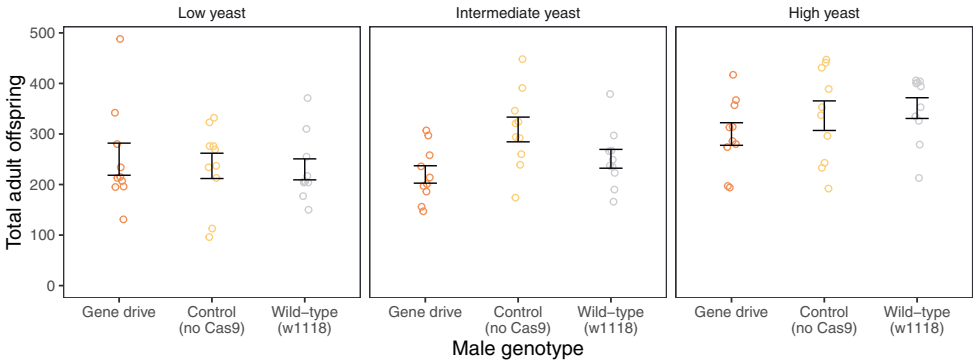


Figure 3.5. Male fertility assay on different yeast concentrations. All drive carriers and controls were heterozygous for the drive. The number of offspring is summed over all seven females paired with one male. Error bars represent the mean and standard error around the mean.

(likely because females were also on this medium for 24 hours during mating, thus mildly impacting fecundity similar to our egg assay in Figure 3.3). Genotype or an interaction between yeast and genotype did not have a significant effect (see Table S3.11 and Table S3.12 for model selection and statistical significance). We conclude that male fertility also did not significantly contribute to the fitness cost observed in our population study.

3.3.7 Viability and ageing

Next, we tested the viability of adult flies directly, and that of eggs and larvae indirectly. The second model fitted to our population study included a viability effect (Table 3.2), as it may be a significant determining factor for drive carrier fitness. We measured whether life-span was different between gene drive and control flies, potentially indicative of subtle fitness cost due to somatic expression or other off-target activity of the gene drive (Langmüller et al., 2022). Furthermore, we indirectly measured whether the survival of larvae with a gene drive genotype might be affected, based on the fact that Cas9 inheritance should be 50% irrespective of whether the offspring also inherit the gene drive or not. Therefore, we can measure discrepancies between number of offspring that inherit Cas9 with and without the gene drive. We tested these factors on intermediate yeast, although repeating it in all three environments would be interesting.

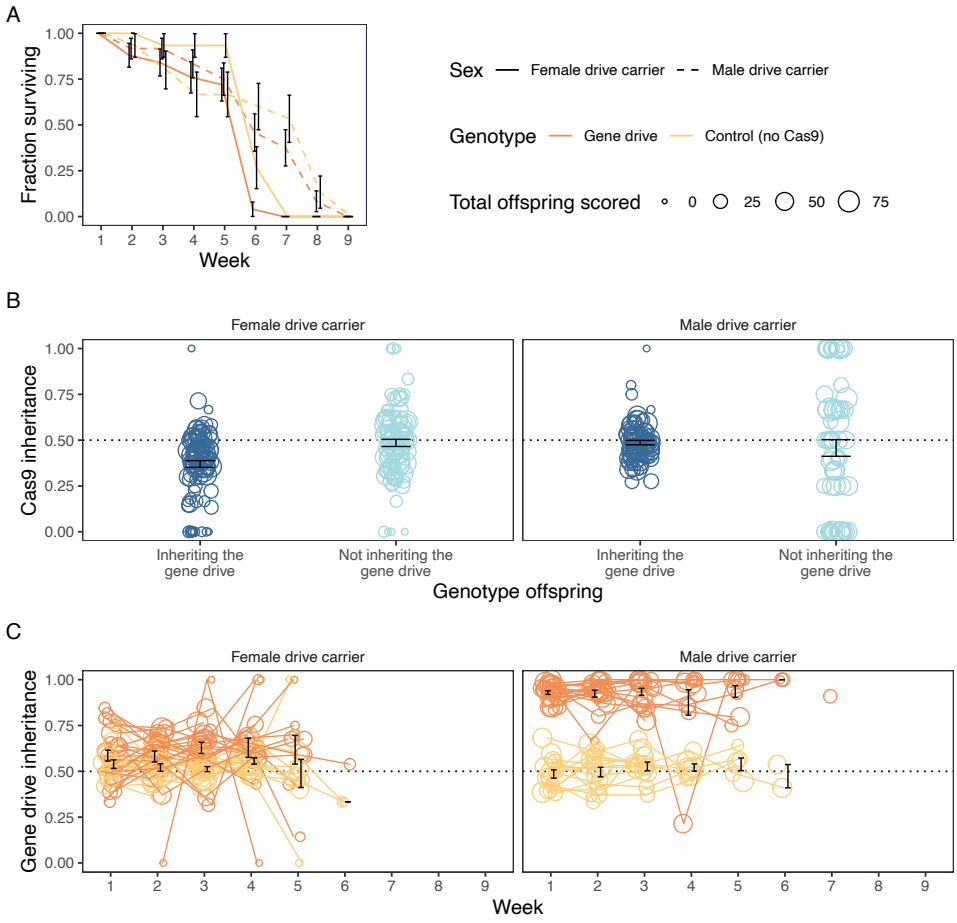


Figure 3.6. The effect of gene drive on viability and ageing. A) Lifespan of drive and control flies, B) an indirect measure of offspring viability based on both parental and offspring genotype, and C) gene drive inheritance rate over the lifetime reproduction. All drive carriers were heterozygous for the drive. Error bars indicate the standard error of the mean. This assay was performed in an intermediate yeast environment only. Error bars represent the mean and standard error around the mean.

We found that there is no clear difference between the survival of gene drive and control groups, but it should be noted that our sample size was quite small (25 gene drive flies and 15 control flies) (Figure 3.6A and S3.2). However, there was a marginally significant

difference between the lifespan of males and females (see Table S3.13 and Table S3.14 for model selection and statistical significance). Overall, the lifespan of our flies was perhaps slightly shorter than is usual for w^{1118} flies (Ziehm et al., 2013). This reduced lifespan could be due to the construct, with or without Cas9 present, leading to reduced survival, but it could also be differences in "local" rearing conditions. Unfortunately, we did not include in our experiment w^{1118} flies without the gene drive construct. Interestingly, female drive carriers seem to live shorter than male drive carriers, with a precipitous decrease after the 5-week mark. This difference between males and females contrasts with existing literature, where females usually live longer than males (Ziehm et al., 2013). Therefore, this result could be indicative of females experiencing reduced viability due to the gene drive construct (but not necessarily an active one). It should be noted that although males live longer, they usually are not able to reproduce any more after week 5 (Figure S3.3). This result is mostly interesting from a theoretical point of view, as flies did not live this long in our cage studies, and likely also would not in the wild.

Also visible in Figure S3.3 is that female drive carriers produce significantly fewer offspring compared to control females (Figure 3.1, see Table S3.15 and Table S3.16 for model selection and statistical significance). This result mirrored our initial observations of slightly reduced population sizes in our cage experiment (Figure 3.1) and slightly reduced egg laying rates and egg-to-adult survival rates for drive individuals (Figure 3.3). This result further confirmed that female fecundity suffered from some drive activity, whether it be germline resistance, embryo deposition, or otherwise.

Because of the large amount of offspring in this experiment and the use of a split drive, we could investigate the inheritance of each individual component of the gene drive (the construct and Cas9 in all four combinations) with high accuracy. We observed that, although Cas9 should be inherited at 50%, regardless of whether the gene drive is inherited along with it, it is actually inherited significantly less when in combination with the gene drive (Figure 3.6B). This effect is visible primarily for female drive carrying parents. This effect is likely not due to maternal deposition, as this would affect all offspring equally. Somatic expression and subsequent decreased egg and/or larval survival are also unlikely to be the driving force of this phenomenon, as it would affect male drive parents equally. Perhaps a combination between embryo deposition and subsequent somatic expression in drive inheriting offspring could be causal, but this hypothesis warrants further investigation. When plot-

ting the data from Figure 3.3 in the same manner, but including the three different yeast environments, this effect is also visible, but with lower accuracy (due to lower offspring numbers) and seemingly different directions of effects between environments and drive carriers (Figure S3.4). These data indicate that higher yeast medium might alleviate some of this fitness costs associated to both parental and offspring genotype.

There was no clear increase or decrease in gene drive inheritance over time (Figure 3.6C), contrary to a previous finding that found more homology-directed repair (HDR) in older flies (A. D. Wang & Agrawal, 2012). However, few flies survive until the later weeks in our experiment, so we cannot exclude that an effect in the same direction (more HDR in older flies) could be observed with more replicates.

3.3.8 Development time

The final factor that we tested as potentially causal to our drive failure was to measure whether the development time of gene drive flies might be longer. We conducted an experiment to measure development time of each genotype (gene drive heterozygotes, control heterozygotes, and w^{1118}) on each yeast concentration. However, here, we encountered significant problems with differing egg laying rates between the parental crosses: w^{1118} flies laid much more eggs in 24 hours (303 and 264 eggs in two replicate cages) compared to homozygous gene drive females, whether mated to Cas9 males to obtain gene drive heterozygotes (154 and 151 eggs) or w^{1118} males to obtain control heterozygotes (40 and 64). This result may indicate that the fitness cost associated with the (inactive) gene drive construct is largely recessive. It is unclear why the wildtype males produced significantly fewer offspring compared to the Cas9 males, but it is likely due to a batch effect.

Despite the poor egg laying, and thus, poor replication of our development time measures, we observed that gene drive individuals developed significantly slower from egg to adult than either control or w^{1118} "wild-type" flies (Figure 3.7). Genotype has a significant effect on development time (see Table S3.19 and Table S3.20 for model selection and statistical significance), but not yeast or an interaction. However, due to the poor egg laying altogether, Figure 3.7 may show the potentially combined effects of delayed egg laying and longer development time. In the w^{1118} group, which is the best replicated group, we observe that primarily the variance in development time increases with decreasing yeast availability. All in all, further investigation of this phenomenon is warranted with better replication.

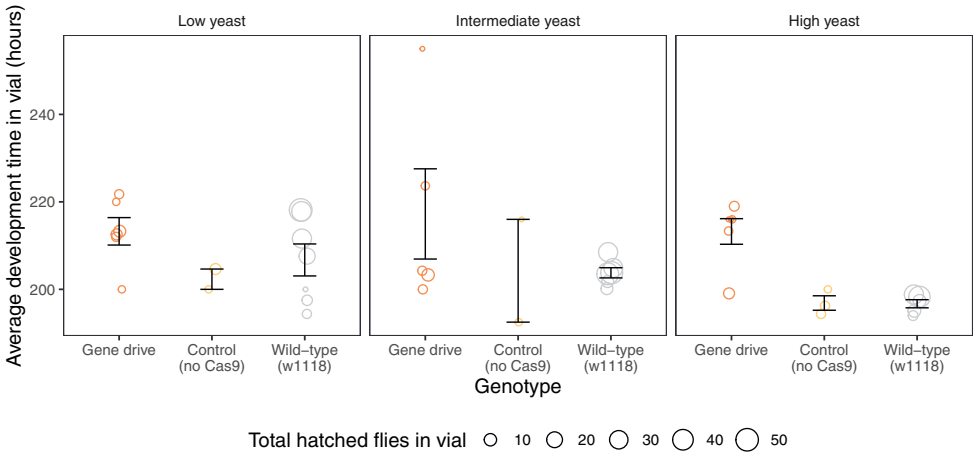


Figure 3.7. Development time of the three genotypes on the three yeast concentrations. All drive carriers were heterozygous for the drive. Error bars represent the mean and standard error around the mean.

3.3.9 High gene drive introduction frequency cage study

The above experiments indicate various life history aspects that may have contributed to a low gene drive efficiency in our cage experiments and how yeast levels may modulate this. Furthermore, as genotype fitness is relative to the presence and frequency of competing/other genotypes (Deredec et al., 2008), we repeated the cage study with two different introduction frequencies, 33% and 66%, on both intermediate and high yeast levels (as these two environments showed the strongest yeast effect; Figure 3.1). Homing modification gene drives can be frequency-dependent in a specific set of parameters, as high gene drive fitness cost can theoretically be offset by an even higher introduction frequency (J. Champer, Lee, et al., 2020). More importantly, a threshold for spread can appear when the fitness cost related to the gene drive is at least partially dominant or when there is a heterozygote disadvantage over either homozygote (Deredec et al., 2008).

Although this and the previous cage experiment were conducted at different times with different batches of flies, previous results were replicated quite closely (Figure 3.8). Again, there is a clear difference in gene drive efficiency between intermediate and high yeast concentrations at 33% introduction frequency (see Table S3.17 and Table S3.18 for model selection and statistical significance). More interestingly, in high yeast medium at the higher

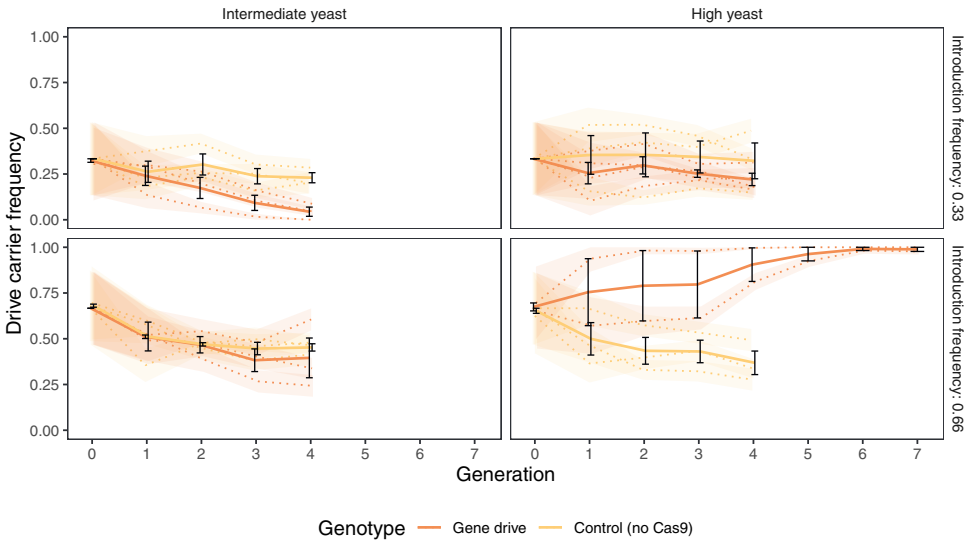


Figure 3.8. Cage study of the gene drive in fruit fly populations on different yeast contents and with two introduction frequencies. Gene drive carrier frequency over time, where the dotted lines represent the three population replicates with translucent ribbons that indicate the standard error around the mean, whereas the solid line and error bars represent the mean and standard error around the mean over the three replicates. Unfortunately, one replicate from the gene drive group introduced at 0.66 frequency in the high yeast concentration was accidentally initiated at 0.33 instead.

introduction frequency of 66%, the gene drive is now able to increase in frequency in both replicates. Note that although we kept the two replicates in high yeast medium to track drive efficiencies for an extra three generations, one replicate in intermediate yeast medium also showed an increase in generation four that might have led to eventual gene drive fixation.

At higher introduction frequency, another notable result we observed was that the control construct now decreased in frequency, instead of lingering at the initial frequency. This result indicates a fitness cost related to the presence of the inactive gene drive construct, instead of merely a fitness cost related to gene drive activity. As this fitness cost did not manifest itself at lower frequencies, we hypothesize that the cost is likely recessive, and thus

limited to homozygous carriers of the construct. However, the biggest decline in frequency occurs in the first generation, when all individuals are still heterozygous for the drive. In any case, this result is in line with the egg laying problems during the development time experiment and anecdotal observations (but consistent over roughly 50 generations) during routine maintenance of this homozygous line. This line has always reproduced very poorly, most notably due to poor egg laying, especially when using older medium that was stored in the fridge for some weeks. The exact cause of this fitness effect is unknown, but the recoded *RpL35A* sequence may somehow slow down protein production during development or reproduction (Hopes et al., 2022).

3.3.10 Performance of another gene drive across yeast environments

Finally, we have measured total reproductive output for another, similar gene drive: the same *RpL35A* gene drive but with an additional distant-site target and with *nanos* Cas9 instead (Faber et al., 2024). Similarly, we find that the number of adult offspring depends strongly on the yeast concentration in the environment of the parents and subsequent developing offspring (Figure S3.5A). The same is true when only the yeast concentration during the larval development of the drive parents is varied (Figure S3.5B). In general, for female drive carriers, the number of offspring remained stably low across environments, likely due to maternal deposition of Cas9/gRNA resulting in non-functional resistance allele formation, which renders offspring non-viable (Faber et al., 2024). For male drive carriers, the number of offspring was much higher and more variable in an expected manner: the higher the amount of yeast in the medium during egg laying, the more offspring and the higher the yeast concentration during the development of the parents, the fewer offspring, likely due to unhealthier parents. At the same time, gene drive inheritance is remarkably stable across all experimental conditions, as well as in ageing flies on intermediate yeast (Figure S3.6).

3.4 Discussion

Gene drives are conceptually powerful tools for population modification or suppression (Bier, 2022), yet their practical application remains largely unproven, particularly under the complex and fluctuating conditions of natural environments (Connolly et al., 2024). To date, most gene drive studies have been conducted in stable, homogeneous laboratory

settings, where environmental variability is minimized. Although substantial effort has been devoted to investigating the basis of mechanistic fitness costs such as embryonic deposition of gene drive components (J. Champer et al., 2017; Guichard et al., 2019; Verkuijl et al., 2022), the influence of environmental factors has received little attention.

In this study, we have demonstrated that even altering a single environmental variable—the amount of yeast in the medium for *Drosophila melanogaster*—can significantly affect the efficiency of a gene drive in a population. To explore the underlying causes of this result, we conducted a non-exhaustive series of experiments across these tested yeast environments, assessing both gene drive inheritance rates and a number of major fitness-related traits. Our results showed that gene drive inheritance rates remained remarkably stable across environments and over time as flies age. This suggests that environmental effects are more likely to influence fitness components of the gene drive carriers than inheritance rates themselves (A. D. Wang & Agrawal, 2012).

Overall, we did not identify a single dominant factor driving the observed failure of the gene drive in our population experiments. Instead, we found a range of subtle effects: gene drive females laid slightly fewer eggs compared to w^{1118} "wild-type" females, egg survival rates were marginally reduced and larval survival was likely also impacted, female viability was unusually lower than males, and development time was perhaps modestly extended. We also found evidence suggesting that at least some of the fitness costs were specific to drive heterozygotes, causing frequency dependent dynamics, and that some fitness costs were likely recessive. Together, these findings indicate that environmental variation exerted diffuse, but cumulatively relevant influence on gene drive carrier fitness. Importantly, the factors tested in our experiments are likely only a subset of the factors at play. Future studies could consider testing additional parameters such as homozygous *versus* heterozygous fitness, the exact impact of parental and grandparental genotype effects, and the relative contributions of larval *versus* adult environmental conditions to any observed effect. Furthermore, all of these life-history traits could be measured in bottles and in vials, as many fitness factors may be considerably different between the two. Density-dependence could thus be an important factor to test explicitly, as increased competition for resources may reveal fitness costs that are otherwise invisible.

When we released the gene drive at high frequency, it was able to successfully spread, indicating that this gene drive is threshold dependent, an unexpected finding for a hom-

ing modification gene drive (J. Champer, Lee, et al., 2020; Deredec et al., 2008). This threshold-dependence is likely a result of drive heterozygote-specific fitness cost: the creation of lethal genotypes in the germline due to inefficient homing, and fitness costs associated with maternal deposition and/or somatic drive expression. These effects essentially constitute a dominant fitness cost in heterozygotes that is absent in either homozygote (wild-type or drive), thus mimicking underdominance gene drives (J. Champer, Champer, et al., 2021).

More broadly, our work underscores how limited our current understanding is of gene drive performance outside the laboratory. If even a single variable such as yeast content can alter drive dynamics, the complexity of real-world environments—where temperature, resource availability, predation, parasitisation, humidity, and competition fluctuate constantly—poses a far greater challenge. It should be noted though that yeast is an extremely important resource for *D. melanogaster*, as it provides them with most of their required nutrition, so not all other environmental factors might prove equally relevant in this species (Ashburner, 1989). A recent field trial with non-gene drive, genetically modified mosquitoes found that fitness and dispersal were reduced compared to wild-type individuals, even without the added impacts of an active gene drive (Yao et al., 2022). Arguably, together these findings highlight the need for contained outdoor trials before larger-scale releases into open environments. For example, experiments in contained, natural conditions such as field mesocosms constructed for *Drosophila* evolutionary studies could be a valuable intermediate step (Rudman et al., 2022). Measuring gene drive carrier fitness directly in realistic settings will be essential, as laboratory conditions may both conceal and exaggerate certain effects. For example, slightly slower developmental rates may be inconsequential in the wild, where generations overlap and food is continuously competed for, but detrimental in the laboratory's rigid, discrete generation cycles. Conversely, laboratory confinement may underestimate mating competition, viability, or dispersal dynamics that would be critical in natural populations.

In summary, our findings represent an initial step towards understanding how environmental variation influences gene drive efficacy. Although our results are specific to *Drosophila*, a fast-developing insect with distinctive life history traits, they highlight the broader importance of evaluating gene drives under ecologically relevant conditions across species. Premature open field releases risk not only the failure of current gene drive con-

structs, which may well be expected given most gene drives' present limitations, but, more importantly, a loss of public trust that could hinder future development of the technology. A cautious, data-driven progression from controlled variability to real-world complexity is essential to ensure that gene drive development advances responsibly, whether toward construct success or failure, without leaving behind what could be considered genetic "space junk": elements unlikely to be harmful, yet perhaps undesirable from philosophical and practical perspectives, as they may interfere with future, more refined gene drive designs.

3.5 Methods

3.5.1 Split gene drive, fly crosses, and phenotyping

For these experiments we chose to use a highly efficient split gene drive: the haplolethal homing gene drive located in the ribosomal subunit gene *RpL35A* (J. Champer, Yang, et al., 2020). For Cas9 and promoter, we chose one of the most efficient promoters from Du et al. (2024), namely the Cas9 under the *CG4415* promoter (belonging to the gNacα gene, a germline-specific gene involved in ribosomal binding activity) with a *nanos* 5'UTR (line SNc9XnGr). This promoter drives highly efficient germline conversion in male carriers, and less so in female carriers. The benefit of this reduced activity in females is that embryo deposition of gene drive components is also limited. Limiting embryonic gene drive activity is especially important in a haplolethal gene drive, because any induced non-functional resistance alleles are likely highly deleterious.

Both elements of the split drive contain fluorescent markers in the construct. The gene drive element contains DsRed under de eye-specific promoter *3xP3*, and the Cas9 element contained EGFP also under the *3xP3* promoter. Flies were phenotyped under an AxioZoom V16 fluorescence binocular (Zeiss, Oberkochen, Germany).

Flies heterozygous for both the gene drive and Cas9 for all experiments were obtained via single crosses of gene drive females with Cas9 males. For the controls, w^{1118} "wild-type" males were used instead, making the drive and control flies maternal half-sibs to control batch effects. Thus, Cas9 was supplied paternally to avoid embryonic deposition of Cas9 in females in the first generation. Only for the second set of cage experiments were split drive flies crossed to Cas9 flies again to obtain flies homozygous for Cas9. For all experiments,

initial crosses were performed on our intermediate yeast medium.

3.5.2 Fly rearing and medium

All initial fly crosses were performed in vials with 6 mL of standard medium containing yeast (70 gr/L), sugar (100 gr/L), and agar (20 gr/L), with nipagin (15 mL of 100 gram/L EtOH) and propionic acid (3 mL of 13.39 M) to avoid bacterial or fungal contamination. For flies on "high" yeast, we doubled the amount of yeast in this recipe, while for the "low" yeast groups we halved it (140 and 35 gr/L, respectively). Flies were kept at 25°C on a 16:8 hours cycle of light:dark. Handling of flies happened under CO_2 sedation. Experiments were done in compliance with Dutch genetically modified insect regulations.

3.5.3 Cage studies

For our cage studies, we kept the flies in glass bottles with 60 mL of the three types of above-mentioned medium. Egg laying was done for 24 hours in fresh bottles from Thursday to Friday, after which the adult flies were frozen for phenotyping. The eggs developed into adult flies over 10 days and were transferred to fresh bottles after hatching on Monday. Three days later, fresh bottles were supplied for egg laying again. This cycle was repeated for five generations for the first set of cage experiments, and for four generations in the second set (with the exception of two replicates which were transferred for three extra generations).

For the first set of cage experiments on the three medium types (low, intermediate, and high yeast), the gene drive was introduced into a population of homozygous Cas9-bearing individuals so the gene drive could keep spreading despite it being a split drive. The gene drive individuals themselves were heterozygous for the gene drive and Cas9, so Cas9 was not entirely fixed but instead at 83,3% frequency. Generation 0 consisted of 24 virgin females homozygous for Cas9, 8 males homozygous for Cas9, and 16 males heterozygous for the gene drive and Cas9. Therefore, the initial introduction frequency of the gene drive was 16,7%, and the gene drive carrier frequency was 33,3%.

The second set of cage experiments on two medium types (intermediate and high yeast) was performed in exactly the same manner with two exceptions; due to the higher introduction frequencies, we opted to introduce individuals that were homozygous for Cas9 instead (but still heterozygous for the gene drive). We also introduced male and female

drive carriers in equal ratios due to the higher introduction frequencies. Therefore, Cas9 frequency was 100%, and gene drive introduction frequencies were 16,7% and 33,3%. Drive carrier frequency was thus 33,3% and 66,7%, respectively. Generation 0 consisted of 8 virgin Cas9 females, 4 virgin gene drive females, 8 Cas9 males and 4 gene drive males for the lower introduction frequency cages. For the higher introduction frequency cages, generation 0 consisted of 4 virgin Cas9 females, 8 virgin gene drive females, 4 Cas9 males and 8 gene drive males.

For both sets of cage experiments, control populations were kept alongside the split drive populations with exactly the same conditions and gene drive introduction frequencies, except that Cas9 was absent from both the split drive flies and the cage populations. We used w^{1118} "wild-type" flies in crosses and cage introductions instead.

3.5.4 Female fecundity assay

To investigate the fertility of female drive carriers, flies heterozygous for the gene drive (either male or female carriers, both with and without Cas9 as control) were paired with a single w^{1118} partner in vials containing 6 mL of the three medium types (low, intermediate, or high yeast). To account for batch effects as much as possible, as fecundity is a highly variable trait, parental crosses were spread over up to 20 separate vials, resulting in roughly equal densities during the larval development stage. Flies used for the crosses were also of similar equal age between groups (between one to four days old). Flies were allowed to lay eggs in fresh vials for 24 hours for five consecutive days, and after those 24 hours, eggs were counted under a binocular. After roughly 10 days of development, adult offspring was counted to calculate the egg-to-adult survival rate, and offspring was phenotyped for fluorescence to determine gene drive inheritance.

3.5.5 Mate choice assay

To see whether gene drive males are equally competitive compared to w^{1118} "wild-type" mates in a cage population, we performed a mating competition assay. This experiment was done only on intermediate yeast, as flies seemingly did not interact with the medium until a mate was selected. Seven-day old virgin w^{1118} females were put in glass bottles containing 60 mL of intermediate yeast medium, and two virgin males of roughly the same age were added: one w^{1118} male and one gene drive (heterozygous for the gene drive and Cas9)

or control male (heterozygous for the gene drive but without Cas9). Flies were monitored until the first mating, which usually occurred within 15 minutes. Male flies were phenotypes for fluorescence to determine the chosen mate.

3.5.6 Male fertility assay

To determine whether the fertility of drive-carrying males was lower than w^{1118} "wild-type" males, we performed a male fertility assay. Virgin drive carrying males (heterozygous for the drive and Cas9), control males (heterozygous for the drive but without Cas9), and w^{1118} "wild-type" males (neither carrying a drive or Cas9) were each provided with seven virgin w^{1118} females for 24 hours in vials containing 6 mL of low, intermediate, or high yeast medium. Males were kept on their respective medium types since after hatching. All flies were between four to seven days old. After 24 hours, males were removed and females were put in individual vials and allowed to lay eggs for five days. After roughly 10 days of development, offspring was counted.

3.5.7 Survival and ageing

To test the fitness of drive carrying individuals and the effect of ageing on drive efficiency, we tested the lifespan and the efficiency of the gene drive inheritance over that lifespan. We tested both drive and control flies (again, heterozygous for the drive and Cas9, or only carrying the gene drive without Cas9 for the control), and also male and female drive carriers. Single crosses were made in vials with 6 mL of intermediate yeast medium, and every week until the flies died, drive or control flies were transferred to fresh vials with new w^{1118} partners. Offspring were frozen and phenotyped for fluorescence.

3.5.8 Development time

Egg laying was done in cages on Petri dishes containing intermediate yeast fly medium. Extra live yeast was provided to stimulate egg laying. Parental crosses were made in two groups for each of our tested genotypes, with roughly 25 females and males each per group. After 8 hours, egg laying checked, after which it was determined that not enough eggs had been laid. Therefore, egg laying was extended a further 16 hours, making 24 hours in total. Eggs were rinsed of the Petri dishes with PBS and transferred to a tube using a brush and funnel. Eggs were then distributed to vials at roughly equal density by transferring 6 µL of

PBS and eggs using a cut-off pipetting tip.

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Author contributions

This work was conceptualised by N.R.F., in collaboration with B.J.Z., J.H., B.A.P., and J.C.. Experimental works was conducted by N.R.F., R.M., T.T., B.B., R.K., and M.G.. Statistical analyses were conducted by N.R.F., except the fitness estimate analysis, which was conducted by R.F. and J.C.. Writing was primarily done by N.R.F., with assistance from J.C., J.H., B.A.P., and B.J.Z., and all authors read and reviewed the manuscript.

3.6 Supplementary material

Table S3.1. Model selection for gene drive frequency in the first set of cage experiments (Figure 3.1). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	4	−202.73	−192.04	105.37	−210.73				
m_full	39	−248.02	−143.78	163.01	−326.02	115.29	35	1.691×10^{-10}	***

Table S3.2. Significant effects in the best-fitting model for gene drive frequency in the first set of cage experiments (Figure 3.1). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
yeast	5.7106	2	0.057 54	.
genotype	21.3824	1	3.762×10^{-6}	***
generation	97.9656	5	$<2.2 \times 10^{-16}$	***
yeast:genotype	5.1573	2	0.075 88	.
yeast:generation	15.6639	10	0.109 67	
genotype:generation	51.4161	5	7.107×10^{-10}	***
yeast:genotype:generation	20.1748	10	0.027 64	*

Table S3.3. Model selection for the total gene drive inheritance rate (Figure 3.3C). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	1	985.88	988.51	−491.94	983.88				
m_reduced	4	480.21	490.71	−236.10	472.21	511.677	3	$<2 \times 10^{-16}$	***
m_full	12	485.11	516.61	−230.55	461.11	11.098	8	0.1962	

Table S3.4. Significant effects in the best-fitting model for the total gene drive inheritance rate (Figure 3.3C). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
genotype	221.050	1	$<2.2 \times 10^{-16}$	***
drive_parent	35.632	1	2.384×10^{-9}	***
genotype:drive_parent	127.196	1	$<2.2 \times 10^{-16}$	***

Table S3.5. Model selection for population size in generation two (the generation after which logistic growth ends) in the first set of cage experiments (Figure 3.1). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	2	256.48	258.26	−126.24	252.48				
m_full	7	229.19	235.42	−107.59	215.19	37.297	5	5.221×10^{-7}	***

Table S3.6. Significant effects in the best-fitting model for population size in generation two (the generation after which logistic growth ends) in the first set of cage experiments (Figure 3.1). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
yeast	111.9765	2	$<2 \times 10^{-16}$	***
genotype	6.8792	1	0.008 72	**
yeast:genotype	6.0861	2	0.047 69	*

Table S3.7. Model selection for the total amount of eggs laid (Figure 3.3A). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	2	838.16	843.41	−417.08	834.16				
m_reduced	5	780.82	793.95	−385.41	770.82	63.336	3	1.138×10^{-13}	***
m_full	13	784.34	818.46	−379.17	758.34	12.483	8	0.1309	

Table S3.8. Significant effects in the best-fitting model for the total amount of eggs laid (Figure 3.3A). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
yeast	65.551	2	5.832×10^{-15}	***
drive_parent	16.058	1	6.144×10^{-5}	***

Table S3.9. Model selection for the total egg-to-adult survival rate (Figure 3.3B). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	2	−97.623	−92.373	50.811	−101.62				
m_reduced	8	−99.671	−78.671	57.835	−115.67	14.0478	6	0.029 11	*
m_full	13	−91.818	−57.693	58.909	−117.82	2.1473	5	0.828 41	

Table S3.10. Significant effects in the best-fitting model for the total egg-to-adult survival rate (Figure 3.3B). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
genotype	6.2092	1	0.012 71	*
yeast	1.8670	2	0.393 18	
drive_parent	0.6848	1	0.407 95	
yeast:drive_parent	6.2561	2	0.043 80	*

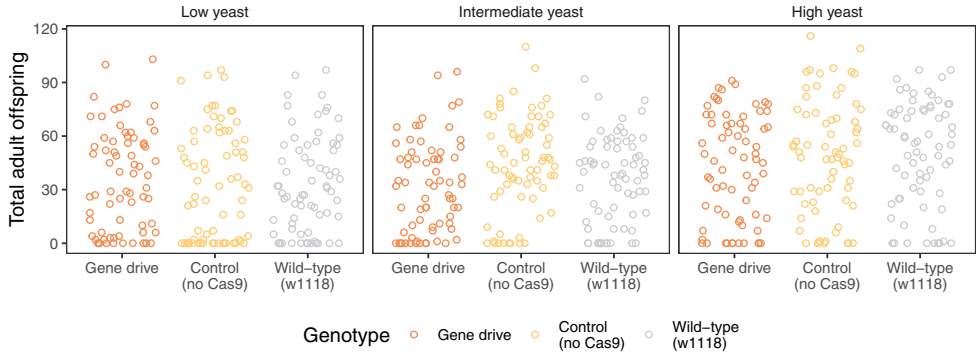


Figure S3.1. Male fertility assay, number of offspring produced per female. All drive carriers and controls were heterozygous for the drive.



Figure S3.2. Week of death of gene drive and control flies and males and females. All drive carriers were heterozygous for the drive. Female drive carriers were given a new w^{1118} male every week and males a new w^{1118} virgin female. Error bars represent the mean and standard error around the mean.

Table S3.11. Model selection for the total amount of offspring per male of varying genotypes (Figure 3.5). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m_null	2	1058.7	1063.7	−527.36	1054.7			
m_reduced	4	1042.0	1052.0	−516.98	1034.0	20.764	2	3.099×10^{-5} ***
m_full	10	1043.4	1068.4	−511.71	1023.4	10.539	6	0.1037

Table S3.12. Significant effects in the best-fitting model for the total amount of offspring per male of varying genotypes (Figure 3.5). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)
yeast	23.355	2	8.485×10^{-6} ***

Table S3.13. Model selection for the week of death of flies of varying sex and genotype (Figure S3.2 and 3.6A). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	2	320.67	325.41	−158.33	316.67				
m_reduced	3	318.82	325.93	−156.41	312.82	3.8509	1	0.049 72	*
m_full	5	320.70	332.55	−155.35	310.70	2.1146	2	0.347 39	

Table S3.14. Significant effects in the best-fitting model for the week of death of flies of varying sex and genotype (Figure S3.2 and 3.6A). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
drive_parent	3.9463	1	0.046 98	*

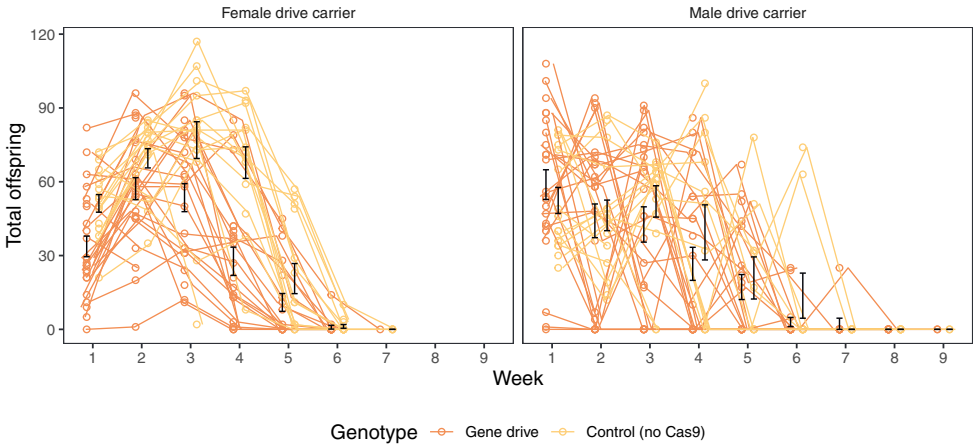


Figure S3.3. Number of offspring produced over the lifespan of gene drive and control flies. All drive carriers were heterozygous for the drive. Female drive carriers were given a new w^{1118} male every week and males a new w^{1118} virgin female. Error bars represent the mean and standard error around the mean.

Table S3.15. Model selection for the total amount of offspring per ageing fly of varying sex and genotype (Figure 3.6). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	4	4494.1	4510.7	−2243.0	4486.1				
m_full	7	4484.5	4513.6	−2235.3	4470.5	15.534	3	0.001 413	**

Table S3.16. Significant effects in the best-fitting model for the total amount of offspring per ageing fly of varying sex and genotype (Figure 3.5). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
genotype	7.1414	1	0.007 532	**
drive_parent	0.5656	1	0.451 995	
genotype:drive_parent	8.3158	1	0.003 930	**

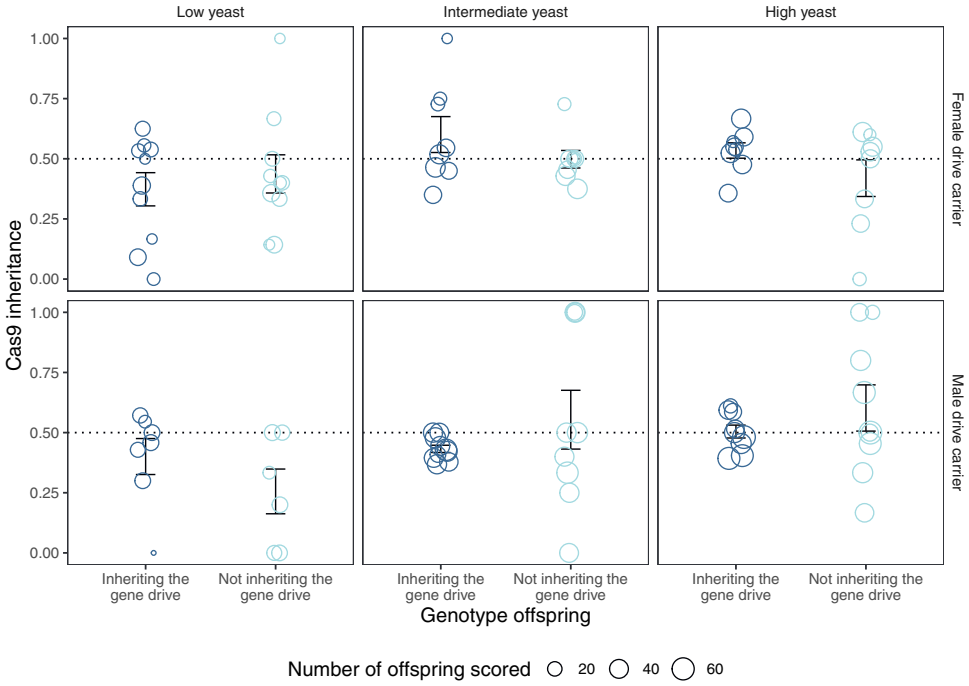


Figure S3.4. An indirect measure of offspring viability based on both parental and offspring genotype on different yeast concentrations. Similar to Figure 3.6B. All drive carriers were heterozygous for the drive. Error bars represent the mean and standard error around the mean.

Table S3.17. Model selection for gene drive frequency in the second set of cage experiments (Figure 3.8). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	4	−177.18	−166.03	92.592	−185.18				
m_full	43	−228.22	−108.35	157.108	−314.22	129.03	39	1.446×10^{-11}	***

Table S3.18. Significant effects in the best-fitting model for gene drive frequency in the second set of cage experiments (Figure 3.8). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
yeast	11.5508	1	0.000 677 2	***
genotype	0.1251	1	0.723 546 4	
generation	62.1659	4	1.017×10^{-12}	***
'Introduction frequency'	140.3452	1	$<2.2 \times 10^{-16}$	***
yeast:genotype	10.4634	1	0.001 217 6	**
yeast:generation	17.0133	4	0.001 921 5	**
genotype:generation	1.9447	4	0.745 933 9	
yeast:'Introduction frequency'	0.5029	1	0.478 216 3	
genotype:'Introduction frequency'	15.6927	1	7.451×10^{-5}	***
generation:'Introduction frequency'	7.6515	4	0.105 213 1	
yeast:genotype:generation	13.2570	4	0.010 085 8	*
yeast:genotype:'Introduction frequency'	7.6522	1	0.005 670 2	**
yeast:generation:'Introduction frequency'	1.9713	4	0.741 040 1	
genotype:generation:'Introduction frequency'	21.7530	4	0.000 224 4	***
yeast:genotype:generation:'Introduction frequency'	11.8038	4	0.018 871 6	*

Table S3.19. Model selection for development time of different genotypes on different yeast concentrations (Figure 3.7). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
m_null	2	328.33	331.81	−162.17	324.33				
m_reduced	4	318.88	325.83	−155.44	310.88	13.454	2	0.001 198	**
m_full	10	326.15	343.53	−153.08	306.15	4.726	6	0.579 404	

Table S3.20. Significant effects in the best-fitting model for development time of different genotypes on different yeast concentrations (Figure 3.7). Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.**

	Chisq	Df	Pr(>Chisq)	
genotype	15.858	2	0.000 360 1	***

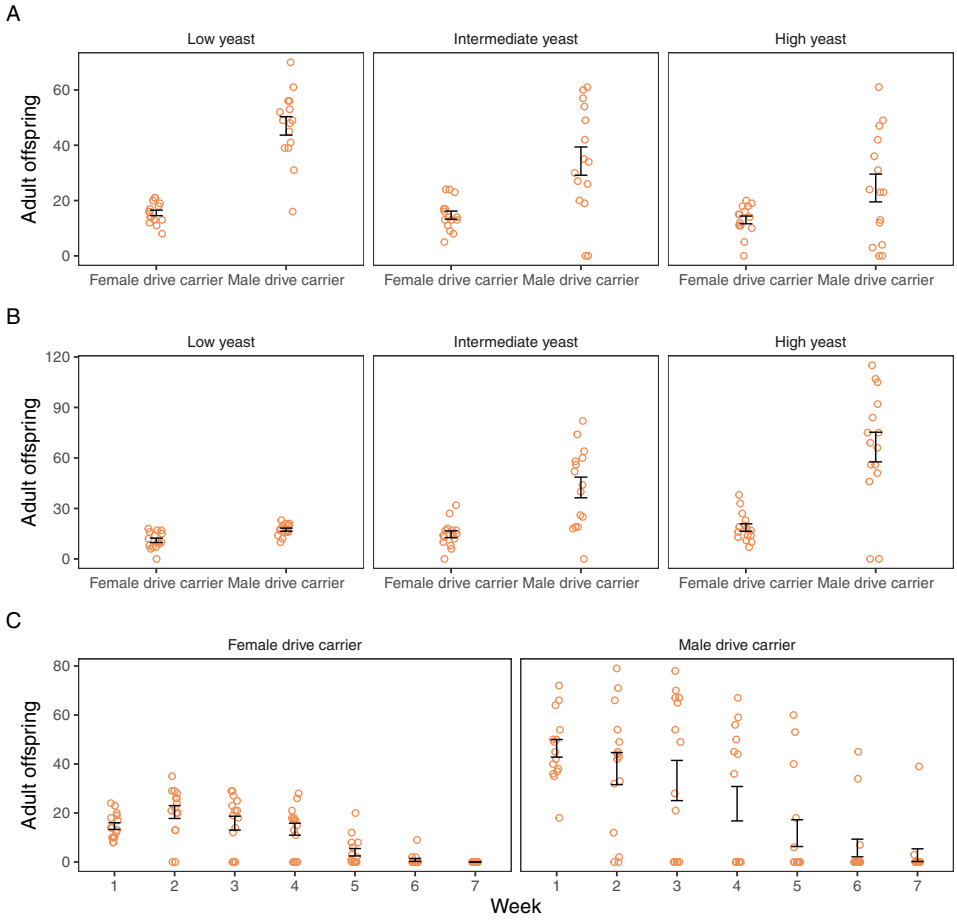


Figure S3.5. Number of offspring of carriers of a different gene drive where yeast was varied in larval diet (A) or adult diet (B), or in ageing flies on intermediate yeast medium (C). All drive carriers were heterozygous for the drive. Error bars represent the mean and standard error around the mean.

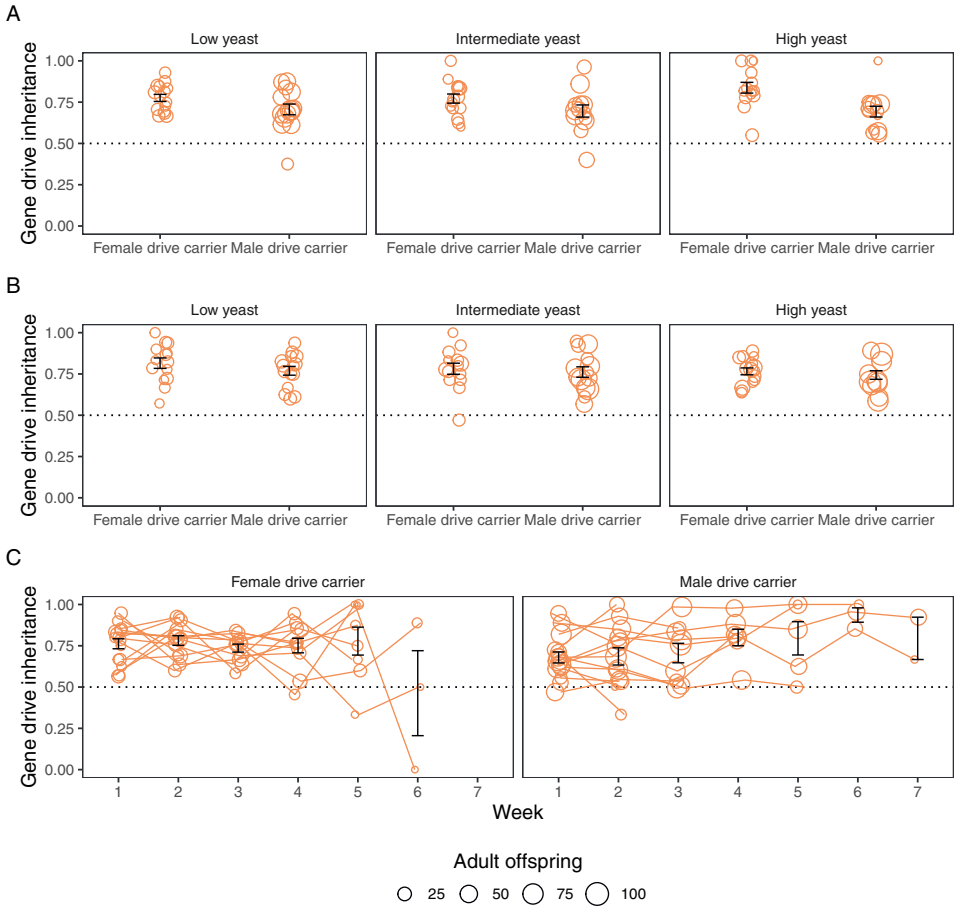


Figure S3.6. Inheritance of another gene drive where yeast was varied in larval diet (A) or adult diet (B), or in ageing flies on intermediate yeast medium (C). All drive carriers were heterozygous for the drive. Error bars represent the mean and standard error around the mean.

""Surfer, wave, same thing."
"That's just stupid," I said. "A surfer's a person. A wave is a wave. How can they be the same?"
Jiko looked out across the ocean to where the water met the sky. "A wave is born from deep conditions of the ocean. A person is born from deep conditions of the world. A person pokes up from the world and rolls along like a wave, until it is time to sink down again. Up, down. Person, wave."
She pointed to the steep cliffs along the shoreline. "Jiko, mountain, same thing. The mountain is tall and will live a long time. Jiko is small and will not live much longer. That's all.""

— Ruth Ozeki, A Tale for the Time Being

Chapter 4

Why don't mice drive? A developmental biology perspective on barriers to homing gene drive efficiency

This chapter is based on:

den Otter, L. R., Pannebakker, B. A., van den Heuvel, J., Zwaan, B. J., Champer, J., & Faber, N. R. (2025). Why don't mice drive? A developmental biology perspective on barriers to homing gene drive efficiency. *SSRN*. https://doi.org/10.2139/ssrn.5702523

4.1 Abstract

CRISPR-based homing gene drives offer a promising strategy for pest management, including combating disease vectors such as malaria-carrying mosquitoes and controlling invasive species. However, their efficiency varies markedly across species. While nearly ideal in *Anopheles* mosquitoes, gene drives show mixed efficiency in *Aedes* mosquitoes and fruit flies, and poor performance in mice. The causes of this variability remain unclear, though the timing of drive activity is likely critical. We believe that variation in gene drive success warrants closer attention from an understudied angle: the developmental biology of the germline. Here, we compare germline development across four species: the fruit fly, two mosquito genera (*Anopheles* and *Aedes*), and the house mouse. Each system offers extensive developmental and genetic knowledge, and multiple gene drive studies in each. We explore how variation in germline formation, timing of gene expression, gonad development, meiosis, and chromosome behaviour may influence drive activity. We argue that considering developmental context during gene drive design can guide future optimisation in both model and non-model organisms. This perspective may help to enable the development of efficient gene drives that can contribute to sustainable invasive pest control in the future.

4.2 Introduction

Gene drives are genetic elements that are inherited at super-Mendelian rates, allowing rapid spread through a population, even when carrying a load with a neutral or negative fitness effect (Bier, 2022). They have been proposed as a useful tool in the fight against vector-borne diseases such as malaria, where they could be used to suppress mosquito populations or to modify them to reduce susceptibility to the malaria parasite (Esvelt et al., 2014). Gene drives could also be used as a more environmentally friendly alternative to control invasive crop pests, reducing the need for insecticides, and provide a humane way of controlling invasive species such as mice and rats (Esvelt & Gemmell, 2017; Esvelt et al., 2014).

There are many different types of natural and synthetic gene drives, of which the CRISPR-based homing gene drive has been widely studied for its efficiency and specificity (Bier, 2022; J. Champer et al., 2016; Werren, 2011). A homing gene drive usually consists of a Cas9/gRNA complex that is expressed in the germline from a specific location in the genome. This complex targets the same allele on the homologous chromosome and creates a double-strand break (DSB) (Figure 4.1A). When this break is repaired through homology-directed repair (HDR), the gene drive is copied into the other allele (this is called homing or drive conversion), making the individual homozygous for the gene drive in its germline cells (Figure 4.1B). Accordingly, the efficiency of a homing gene drive system is calculated as the proportion of offspring in which the wild-type allele has been successfully converted to the gene drive allele. Depending on what the gene drive is designed for, the construct can contain an additional cargo, such as a new or altered gene, or its goal can simply be to disrupt the target gene. However, a homing gene drive is only successful in copying itself if the double-stranded break is repaired through HDR and not through the alternative repair mechanism of end-joining (EJ) (Figure 4.1A), which can include microhomology-mediated end-joining or non-homologous end-joining (Bier, 2022). EJ could lead to the formation of resistance alleles, which have a sequence change at the target site that makes them immune to cutting by the Cas9/gRNA complex (Price et al., 2020). Resistance alleles are one of the main challenges to the effectiveness of current gene drives. They can be especially problematic when the function of the target gene remains intact despite the sequence change. These resistant alleles can lead to strong selection for resistance in the case of suppression drives or low-fitness modification drives (Price et al., 2020).

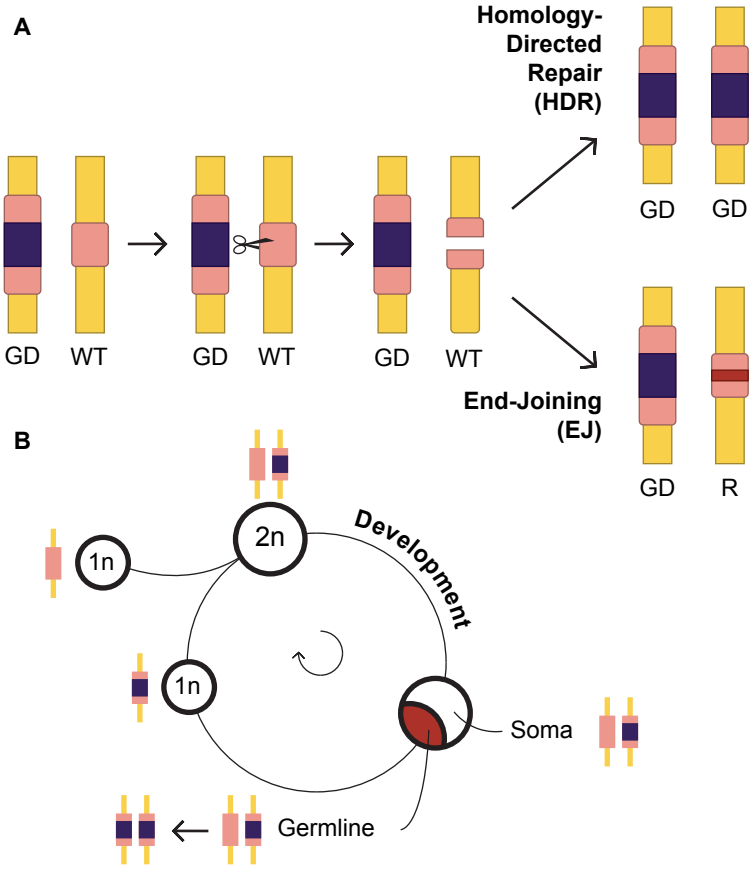


Figure 4.1. Germline gene drive activity. A) The gene drive allele (GD) expresses a Cas9 enzyme in the germline that creates a double strand break in the wild-type allele (WT). This break is repaired either through homology-directed repair (HDR), resulting in a gene drive homozygote (and thus, Super-Mendelian inheritance of the gene drive), or through end-joining (EJ), resulting in a resistant allele (R) B) The cycle through which a homing gene drive spreads in a population by converting wild-type alleles to drive alleles.

For a homing gene drive to be as efficient as possible, it is important that its activity is strictly limited to the germline (M. A. E. Anderson et al., 2023; Du et al., 2024; Hammond, Karlsson, et al., 2021; Pfitzner et al., 2020). This is not only important to achieve a baseline efficiency in order for the drive to spread in a population (E. Yang et al., 2022), but also to avoid the potential formation of resistance alleles due to increased EJ repair instead of HDR (J. Champer et al., 2018). To these ends, suppression and some modification gene drives should limit the expression of Cas9 in the soma to avoid potential fitness costs from disrupting the target gene (Hammond et al., 2016), and all homing gene drives should avoid drive activity in the embryo from maternal deposition, which can be a primary source of resistance alleles (Adolfi et al., 2020; J. Champer et al., 2018). Specifically, for HDR to facilitate gene drive homing, it is considered key that nuclease activity should coincide exactly with meiosis, because at that time, homologous chromosomes are tightly linked for homologous recombination to occur during crossing-over (Pfitzner et al., 2020). This chromosomal arrangement would make HDR the more likely repair mechanism rather than EJ (Bier, 2022). Therefore, developers of homing gene drives use promoters that are active primarily in the germline, such as the promoters *nanos* and *vasa* in *Drosophila melanogaster* (J. Champer et al., 2017), and their orthologs in *Anopheles* mosquitoes (Calvo et al., 2005; Gantz et al., 2015; Papathanos et al., 2009) and mice (Pfitzner et al., 2020).

Interestingly, in these various organisms, significant differences in homing efficiencies have been observed between species, despite fairly conserved germline processes and comparable gene drive designs (Verkuijl, 2023). In *Anopheles* mosquitoes, homing gene drives usually (but not always) reach well over 95% (and not uncommonly 100%) inheritance rates (Adolfi et al., 2020; Carballar-Lejarazú et al., 2023; Hammond et al., 2016; X. Xu et al., 2025). Homing drive inheritance in *Aedes* mosquitoes is much lower, usually ranging between 50% and 75%, despite high Cas9 cleavage rates (M. A. E. Anderson et al., 2023; M. Li et al., 2020; Reid et al., 2022a). For the fruit fly *Drosophila melanogaster*, gene drive inheritance is variable, usually ranging between 60-90% (J. Champer, Yang, et al., 2020; Du et al., 2024; Nash et al., 2022; Oberhofer et al., 2018; Terradas et al., 2021; E. Yang et al., 2022). Experiments with the house mouse *Mus musculus* have shown much lower inheritance rates; usually they are around the standard Mendelian 50% (Grunwald et al., 2019; Pfitzner et al., 2020), though super-Mendelian drive inheritance has been observed both males (5% over Mendelian ratios) and females (up to 22% over Mendelian ratios) (Grunwald et al., 2019; Weitzel et al., 2021). Much remains uncharted regarding why homing gene drives

exhibit markedly higher efficiency in *Anopheles* spp. compared to *Aedes* spp., *Drosophila melanogaster*, and mice, and explanations remain speculative at most.

Here, we offer a perspective on gene drive efficiency from what we believe to be an understudied angle: the developmental biology of the germline. We will discuss two well-studied germline and gene drive promoters, *nanos* and *vasa*, and what is known about their expression pattern during development. Currently, most germline promoters are chosen based on expression patterns in the adult gonads. During development, however, these same promoters might show different activity, and consequently result in unwanted gene drive activity (J. Champer et al., 2018). We take an evolutionary perspective by comparing the germline of *Drosophila*, *Anopheles* and *Aedes* mosquitoes, and, more phylogenetically distant, mice. This comparison will highlight how interspecies differences in gene drive efficiency might relate to variations in the way the germline develops or is regulated. Although germline-specific promoters are somewhat conserved across these four species, it is perhaps too optimistic to assume that this is the only determinant for gene drive efficiency. Fortunately, each of these species offer a wealth of literature on their germline genetics and developmental biology, as well as multiple gene drive papers for each. We argue that differences between species in germline developmental biology and regulation could be linked to gene drive homing efficiency. We explore key differences in germline biology in a chronological order, including mechanisms of germline formation, timing of native gene expression, gonad development, timing of meiosis, and chromosome configuration.

4.3 Germline specification

Many homing gene drives have, in order to be specifically expressed in the adult germline, inadvertently co-opted genes that regulate early embryonic patterning and the initiation of the ancient germline-soma distinction in the embryo. Animals across the tree of life benefit from this early distinction, either because it reduces the number of divisions germline cells have to go through, minimising the chance that mutations build up that are passed along to future generations (Milholland et al., 2017; Weismann, 1893), or as a way to reduce genomic conflict (Buss, 2014). The germline is set apart early on in development when specific cells in an embryo start to differentiate into primordial germ cells (PGCs). How this process is regulated is complex and still an active field of research (Pamula & Lehmann, 2024). In Diptera, the insect order that includes *Drosophila*, *Anopheles*, and *Aedes*, the specification

of the germline is regulated by gradients of maternally deposited factors, including *vasa* and *nanos*, which are also regularly used drive gene drive promoters (Figure 4.2A) (Extavour & Akam, 2003; Reik & Surani, 2015). Contrastingly in mice, PGCs differentiate in response to signals from neighbouring somatic cells, in turn induced by asymmetric extra-embryonic tissues (Figure 4.2B) (Reik & Surani, 2015; Saitou & Yamaji, 2012).

Whether the germline of a species is determined by deposition of maternal proteins or by signalling factors from neighbouring cells immediately marks a relevant difference between species for gene drive functioning due to an unwanted gene drive effect in Diptera: embryonic gene drive activity (J. Champer et al., 2017, 2018; López Del Amo, Bishop, et al., 2020). Many genes that are expressed in the germline, including *vasa* and *nanos*, are maternally deposited into the oocyte to support early embryonic development, even though their zygotic expression is restricted to germ cells. It is therefore unsurprising that *nanos*- or *vasa*-expressed gene drive components are deposited into oocytes as well. In the embryo, DNA repair predominantly occurs via the EJ pathway rather than HDR (Z. Li et al., 2024), making successful gene drive homing unlikely at this stage (Figure 4.1). In experiments with insects, maternal deposition of Cas9 into oocytes has led to problems such as embryonic lethality, somatic mosaicism, and the formation of resistance alleles (Carballar-Lejarazú et al., 2022; J. Champer et al., 2017; Du et al., 2024). The issue with maternal deposition of Cas9 could be exacerbated by the fact that in Diptera, the embryo first develops as a syncytium, which is a single cell with many dividing nuclei (D. Anderson, 1966). This organisation might expose more nuclei to embryonic gene drive activity, as deposited protein and RNA could invade each nucleus, especially when drive components contain nuclear localisation signals (Yadav et al., 2023). Contrastingly in mice, there has been no reported evidence of gene drive activity from maternally deposited factors, despite the use of the *vasa* promoter that has generated high levels of maternal deposition in *Drosophila* and mosquitoes (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021). However, none of the mice drives had the same high germline cleavage rates found in insects and correlated to high embryo activity.

Not only are many transcripts and proteins maternally deposited in Diptera, but some are also localised to the posterior pole of the oocyte by germplasm-collecting granules (Pamula & Lehmann, 2024). These granules are cytoplasmic structures that gather germline determinants (again, including Vasa protein and *nanos* mRNA, Gavis et al., 1996; Liang et

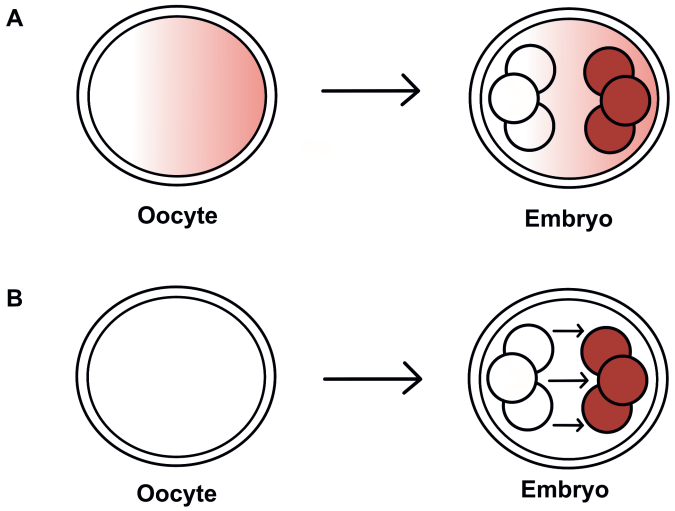


Figure 4.2. Two ways of determining the germline in early zygotes. A) maternally deposited factors form a gradient in the egg cell that determines the fate of cells along that gradient, PGCs (dark red) are specified at the posterior end. B) PGCs are specified in response to signals from neighbouring cells. Dipterans use the mechanism above, mammals use the mechanism below. Figure adapted from Reik and Surani (2015).

al., 1994), ensuring proper germ cell development. Specifically, *nanos* mRNA contains a 3'UTR sequence that binds to these germplasm granules (Gavis et al., 1996). Vasa protein plays an important role in the specification of germline cells (Mahowald, 2001), and acts as a translational regulator for many genes downstream (*nanos* among them) (Saffman & Lasko, 1999). In Diptera, once deposited *nanos* mRNA is translated in the embryo, it is essential for the formation of the anterior-posterior axis and subsequent abdominal development, as well as for germ cell formation (Kato & Nakamura, 2012; Mahowald, 2001). Germplasm-collecting granules could take gene drive components (such as *nanos*-expressed Cas9 mRNA) along as well, potentially causing a disproportionate amount of gene drive embryo activity and subsequent EJ mutations in the first PGCs at the posterior pole (López Del Amo, Bishop, et al., 2020). Conversely, the posterior localisation of drive components has also been suspected to cause "shadow drive" in *Drosophila melanogaster*; a mechanism where homing already happens in the PGCs of the zygote (Guichard et al., 2019). This

would be a beneficial effect for gene drives, though it has not been observed in mosquitoes (Hammond, Karlsson, et al., 2021).

For the design of gene drive constructs that have minimal levels of embryo activity in Diptera, it is important to determine whether it is the maternally deposited gene drive transcripts or proteins that ultimately cause the most EJ mutations, as these would require different optimisation strategies. There are several avenues for construct optimisation: 1) the Cas9 expression window could be chosen so that expression has ceased well before fertilisation of the oocyte (reports in fruit flies showed that different Cas9 promoters could minimize embryo activity while preserving high germline efficiency, Du et al., 2024; Y. Wu et al., 2025), 2) the drive construct could contain extra components so that gene drive mRNA is quickly degraded in the late oocyte (for example, gene drive mRNA expressed by *nanos* could potentially be translationally repressed in the embryo by altering the 3'UTR, Bergsten and Gavis, 1999), or 3) the Cas9 protein could be altered to be less stable in the oocyte or be inactivated by other factors. Currently, it remains unknown which, if any, strategy could most effectively prevent gene drive embryo activity.

4.4 Germline expression and genome accessibility

Across species, the differentiation of pluripotent cells into PGCs involves suppressing the expression of somatic genes (Lehmann, 2012; Reik & Surani, 2015). In *Drosophila*, all transcription is shut down in early PGCs until zygotic genome activation, so they are completely dependent on maternal factors and signals from neighbouring somatic cells (Saffman & Lasko, 1999). This state lasts until their migration towards the primordial gonads after gastrulation, where their own transcription begins. Once zygotic gene expression starts, the translation of somatic mRNAs in PGCs is inhibited by proteins such as Nanos (Asaoka et al., 2019). Contrastingly, in the PGCs of mammals, only genes associated with the somatic fate are suppressed, whereas germline genes are expressed immediately (Reik & Surani, 2015). While there are maternal mRNAs present in developing mammalian oocytes, their degradation begins at the end of meiosis, well before fertilisation. The destruction of maternal factors and the activation of the zygotic genome is complete by the two- to four-cell stage of the embryo (Reik & Surani, 2015). This makes maternal proteins much less prominent in the fertilised egg cells of mammals compared to those of Diptera (L. Li et al., 2010; Sha et al., 2019).

In general, germline cells have distinct expression patterns from somatic cells (Saitou & Yamaji, 2012; Tarikere et al., 2022), which is regulated by transcription factors as well as epigenetic changes, including chromatin remodelling (Hopes et al., 2022; Vidaurre & Chen, 2021). Thus, the expression of the target of a homing gene drive in the germline is expected to influence homing efficiency, as it is important that the target gene is accessible to the Cas9/gRNA complex. For example, in zebrafish, Prill and Dawson (2020) found that genes that had been successfully targeted with HDR were typically also expressed during the time period in which the nuclease was expressed. If the target genes themselves were not expressed in the right time frame, then usually neighbouring genes were (Prill & Dawson, 2020). In mice, the location of gene drive components could be responsible for the low homing efficiencies observed. So far, all germline homing studies in mice targeted the tyrosinase (Tyr) gene, a somatic gene involved in the production of melanin that leads to easily detectable albinism in homozygous loss-of-function mutants (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021). However, genes involved in somatic functions such as body or eye colour are usually targeted without major efficiency impairments in *Drosophila* and mosquitoes (Adolfi et al., 2020; M. A. E. Anderson et al., 2024; Carballar-Lejarazú et al., 2020; J. Champer, Yang, et al., 2020; J. Champer et al., 2017; Gantz & Bier, 2015; Hou et al., 2024). Mice have considerably different chromatin structure and machinery compared to Diptera, so it could be that this is a more important factor in mammals (Szalay et al., 2024). Clearly, the importance of the location of a gene drive and the expression pattern of its target require further systematic investigation in *Drosophila*, mosquitoes, and mice.

In addition to potentially affecting its susceptibility to cutting by Cas9, the location of a gene drive is important because it could affect its expression. Verkuijl et al. (2022) have proposed that targeting a somatically expressed gene might lead to somatic expression due to interactions with cis-regulatory elements or the gene's chromatin context, even when the gene drive itself uses a germline-specific promoter. Experiments with homing gene drives in *Drosophila* have shown that the expression of Cas9, and the efficiency of its associated gene drive, seem to depend partially on the chromosomal location of the promoter-Cas9 construct, although it is unclear what mechanism is causative (Du et al., 2024; Terradas et al., 2021). Terradas et al. (2021) tried several different setups for their *Drosophila* split gene drive (in which Cas9 is expressed from a different locus in the genome than the gene drive) and found that when the promoter-Cas9 complex was inserted into chromosome 3, it ap-

peared to be expressed at higher levels than in chromosome 2 or the X chromosome. Similarly, Du et al. (2024) found that the chromosomal location matters for homing efficiency. Similarly, converting a split drive into a full gene drive with exactly the same components has also been shown to result in differing drive efficiencies (Terradas et al., 2023). This phenomenon could imply that a focus on testing and optimising candidate field-release designs for self-sustaining homing drives is warranted, at least in later stages of testing, despite conflicting with the precautionary principle underpinning laboratory-based gene drive containment strategies (Akbari et al., 2015). This concern is likely not applicable for inherently self-limiting or confined, threshold-dependent gene drive designs.

4.5 From primordial germ cells to gonads

For many current gene drive designs, the developmental stages of the germline before gametogenesis are not explicitly taken into account. However, this could be a key time for unwanted gene drive activity (Figure 4.3) (J. Champer et al., 2017; Grunwald et al., 2019; Verkuijl et al., 2022). For example, after germline specification, both *vasa* and *nanos* are still expressed in PGCs because they are involved in the migration of PGCs toward the gonads, as well as the maintenance of germline stem cells (GSCs) throughout oogenesis (Calvo et al., 2005; Kobayashi et al., 1996; Saffman & Lasko, 1999; Z. Wang & Lin, 2004). Furthermore, germline-specific genes are often also expressed in somatic tissue surrounding the germline, leading to unexpectedly high fitness costs due to EJ events in gene drive heterozygotes Y. Wu et al. (2025) and E. Yang et al. (2022).

Once PGCs have been specified, they migrate towards the location of the embryonic gonads. Along the way, some PGCs are lost (Yamada et al., 2008), and some pick up somatic cells from the mesoderm that will surround the PGCs to form the somatic tissue of the gonads (Dansereau & Lasko, 2008; H. Zhang et al., 2023). In *Drosophila* males, the last stage of embryonic development includes the formation of a stem cell hub, in which germline stem cells surround somatic hub cells that prevent them from differentiating (Le Bras & Van Doren, 2006). In *Drosophila* females, the germline stem cell niche is formed later, during the pupal stage, and includes somatic cap cells that will house the germline stem cells, and terminal filament cells that anchor the germarium (see the later section on oogenesis) (Dansereau & Lasko, 2008). During the larval stages of female *Drosophila*, the PGCs are still dividing. This stage represents one of the few points at which gonad development

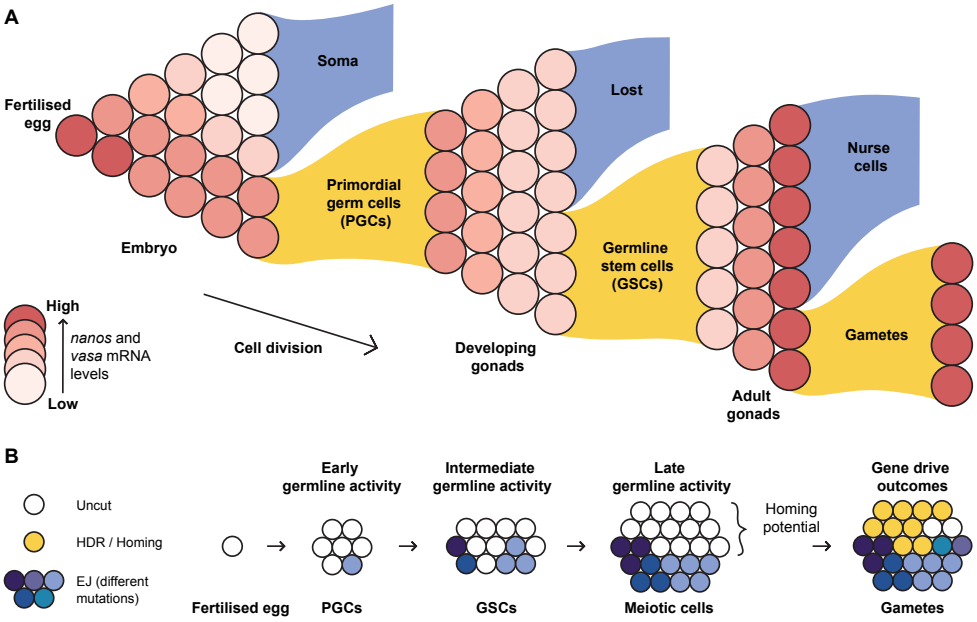


Figure 4.3. Gene drive activity during germline development. A) Illustration showing *nanos* and *vasa* mRNA levels over the full development of the germline. The fertilised egg cell splits into primordial germ cells (PGCs) and somatic cells, after which some PGCs are lost during their migration. Some PGCs become germline stem cells (GSCs) in the adult gonad, and, after a few mitotic divisions, enter meiosis. In females, most of these cells will become nurse cells, and a few will become the gametes to be fertilised and restart the cycle. *nanos* and *vasa* mRNA levels are highly expressed in adult gonads, where they are deposited into the egg cell. Both promoters also maintain low levels of expression during the PGC and GSC stages to avoid premature differentiation of these cells. There is generally no (*nanos*) or low (*vasa*) expression of these promoters in the soma. B) Illustration showing how early or intermediate gene drive activity, during a stage where EJ is the most prevalent repair pathway and the germline consists of only a few PGCs or GSCs, would lead to reduced homing (yellow) potential and more similar EJ mutations (shades of blue) observed in offspring. A mutation in a PGC will be carried by all its daughter cells (PGCs and GSCs), and all their gametes will carry it. Only oocytes or spermatocytes produced from PGCs or GSCs that are still heterozygous wildtype (white cells) during meiosis will undergo independent homing (yellow) or EJ events.

diverges between *Drosophila* and mosquitoes; in *Drosophila* larvae, the PGCs divide independently, whereas in *Aedes* mosquitoes they undergo a synchronised, cyst-like division, meaning that the PGCs stay connected due to incomplete cytokinesis, which appears to be regulated in response to nutrition from a blood-meal (H. Zhang et al., 2023). Another difference in the ovarian development of *Drosophila* and mosquitoes is that, while both contain terminal filament cells during the larval stages, these cells degenerate in mosquitoes at the end of the pupal stage, and are no longer found in adults (H. Zhang et al., 2023). This lack of terminal filament cells in mosquitoes is one of the reasons why the activity of germline stem cells in adult female mosquitoes is debated (H. Zhang et al., 2023). In male mice, PGCs also migrate toward the embryonic gonads, after which so-called Sertoli cells form that surround the PGCs. The PGCs differentiate into pro-spermatogonia and only after birth do they differentiate into spermatogonia and begin spermatogenesis. Spermatogenesis is maintained throughout adulthood by spermatogonial stem cells (Luo & Yu, 2022). In female mice, the PGCs already enter meiosis during the embryonic stage and arrest in meiosis I. Only after birth are the first follicles formed, and eventually the oestrus cycle starts and the first cells complete meiosis (Luo & Yu, 2022).

How these complex developmental stages of the germline impact homing efficiency likely depends on the promoter that is used to express Cas9 (Figure 4.3A). In any species, the current hypothesis is that when the gene drive is expressed before meiosis, the resulting DSBs are more likely repaired through EJ than through HDR (Grunwald et al., 2019). Most attention has been paid to this expression timing during development in the context of mouse gene drives. Both Grunwald et al. (2022) and Pfitzner et al. (2020) discuss that *vasa* is likely expressed too early in male and female mice for HDR to take place, as it is first expressed in PGCs in embryonic gonads (Toyooka et al., 2000), while HDR in mice is limited to later phases of meiosis. However, when Grunwald et al. (2019) used *vasa*-Cre, a modified system with delayed expression compared to normal *vasa* expression, Cas9 expression was timed appropriately for meiosis in females, but still too early in males, where it was active during mitotic divisions instead, and no homing was detected (Grunwald et al., 2019).

There is also some indirect evidence for this hypothesis in insects. Namely, after early drive activity, a PGC or GSC would acquire a unique mutation that it passes on to its daughter cells (Figure 4.3B). The earlier the EJ happens, the more similar mutations should be found

in the offspring. Indeed, a gene drive experiment in *Anopheles* found just four unique mutations in 218 offspring of drive females (whose DSB were repaired through EJ) as opposed to 18 unique mutations in 239 offspring of drive males (indicating that few mutations are possible due to a biochemical phenomenon) (Hammond, Karlsson, et al., 2021). Similar observations were made in *Drosophila* as well (J. Champer et al., 2017, 2018; López Del Amo, Bishop, et al., 2020). Further indirect evidence for potential early germline gene drive activity is the large amount of variance observed in inheritance rates between crosses with exactly the same drive construct (J. Champer, Wen, et al., 2019; Du et al., 2024; Faber, Champer, et al., 2025; Grunwald et al., 2019), a factor not taken into consideration enough. Overall, the timing differences between the various gene drive promoters (and within a single promoter as well, based on stochasticity) can probably influence the potential for homing later on in the germline.

To further optimise the homing efficiency of gene drives, promoter selection should not be based solely on bulk RNA sequencing or single-cell RNA sequencing of adult gonads (M. A. E. Anderson et al., 2023; Du et al., 2024; Y. Wu et al., 2025), but should also consider expression dynamics during germline development. Of course, the limited number of germline cells at most stages of an animal's development, especially in females, presents a challenge for assessing promoter activity in these tissues specifically. However, recent advances in single-cell RNA-sequencing potentially allow the characterisation of transcription during the entire development of the germline. If necessary, germline cells can be enriched for using marker genes such as *nanos* or *vasa* (Rust et al., 2020; Witt et al., 2019), facilitating the characterization of full germline expression profiles that inform the selection of optimal gene drive promoters. Once gene drives with specific promoters are established, perhaps integrated RNA and DNA single-cell sequencing technologies could further provide highly detailed insights into drive expression patterns and DNA repair outcomes (Choi et al., 2020). Alternatively, an inducible Cas9 system responsive to specific stimuli, such as an ingested chemical, could be employed. However, such approaches are unlikely to provide the temporal and spatial resolution required to accurately profile germline drive activity (López Del Amo, Leger, et al., 2020).

4.6 Gametogenesis and meiosis

Additional interesting differences between *Drosophila*, mosquitoes, and mice emerge in the timing of meiosis and the production of oocytes and spermatocytes. Gametogenesis, specifically prophase I of meiosis I, is when gene drive homing is thought to happen, because then homologous chromosomes are paired for meiotic crossing over. However, the moment at which meiosis is initiated is not the same between species and between males and females (Figure 4.4). The timing of gametogenesis and its relevancy for gene drive activity is discussed in detail below, but first we discuss two cell biological phenomena that differ between our studied species as well: somatic pairing of chromosomes and polytene chromosomes.

4.6.1 Somatic pairing

Some Dipterans, including both *Drosophila* and mosquitoes, show a phenomenon called somatic pairing, which is the pairing of homologous chromosomes outside of meiosis (Figure 4.4B). Due to somatic pairing, the homologous chromosomes are closely linked to each other across the entire chromosome during interphase and prophase of mitosis, in both somatic cells and pre-meiotic germline cells (Clements, 2000; McKee, 2004; Mukherjee & Rees, 1970). However, the mitotic pairing of germline cells is not an extension of somatic pairing, because somatic pairing seems to occur in all cells except PGCs and germline stem cells (Rubin et al., 2022). From an evolutionary perspective, somatic pairing may be suppressed in PGCs and GSCs to prevent deleterious mutations from propagating, though it is unclear how common this phenomena is across Dipterans.

Since homology-directed repair is dependent on the presence of a homologous chromosome, one might logically expect more HDR to take place in somatically paired chromosomes. Somatic pairing could explain why HDR is consistently a major repair pathway in mitotic cells in *Drosophila* (Do et al., 2014; Rong & Golic, 2003). Relevant for gene drives, Z. Li et al. (2021) indeed found high levels of somatic drive conversion in *Drosophila*. Thus, somatic cells heterozygous for a gene drive could become homozygous. For gene drives that carry a fitness load, homing in the soma could be detrimental to the carrier individual. Somatic homing is less likely to occur in mammals, because they do not show somatic pairing. In mammals, HDR sometimes happens in the S and G2 phases of the cell cycle in mitotic

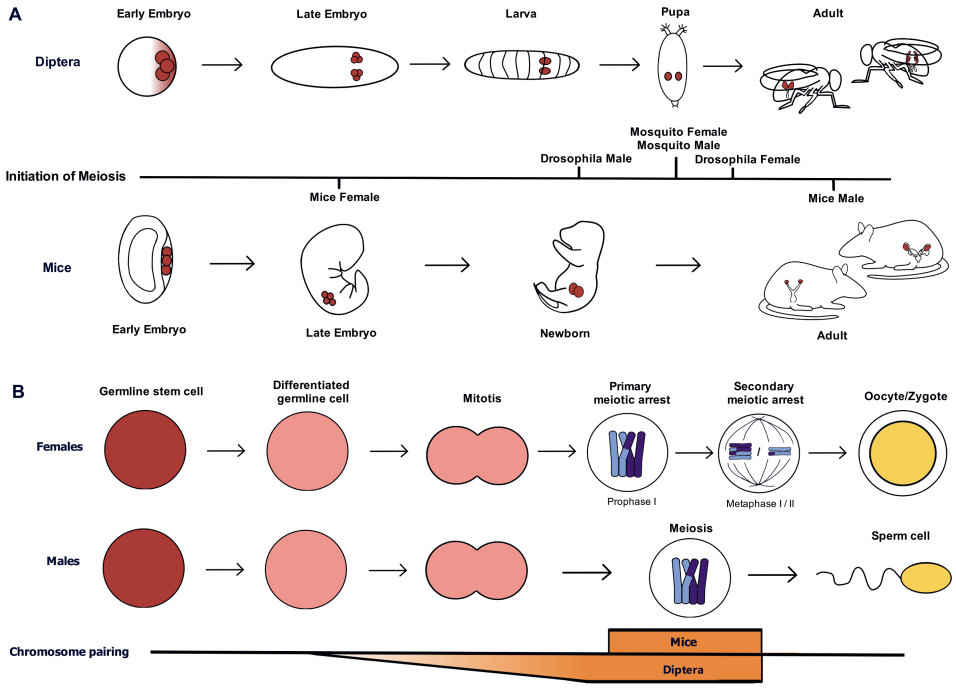


Figure 4.4. Illustration of germline development and the timing of the start of meiosis. A) Germline development and when meiosis is initiated in males and females. B) Illustration of meiosis, indicating how somatic pairing during mitotic divisions in Diptera might allow homology-directed repair (HDR) to take place in more stages of development compared to mice.

cells, and the sister chromatid is typically used as a repair template rather than the homologous chromosome (R. D. Johnson, 2000). This means that even when a DSB was created in somatic cells and repaired with HDR, the sister chromatid would be used to repair the break; thus homing of the drive onto the homologous chromosome would not occur. As said, the more dominant EJ pathway often results in mutations. These somatic mutations can cause a fitness effect, especially in gene drives that target an essential gene (J. Champer, Yang, et al., 2020). So, somatic expression of Cas9 can form a problem in gene drives for *Drosophila*, *Anopheles*, and mice, though the likely repair mechanisms of DSBs and the resulting effects thereof likely differ between gene drives and species.

4.6.2 Polytene chromosomes

Besides somatic pairing, another relevant cell biological difference between species is the presence of polytene chromosomes. Chromosomes can replicate repeatedly outside of mitosis (called endoreplication), and if the chromosomes are not replicated over their entire length but only certain areas, the daughter chromosomes remain together and form one big polytene chromosome. Polytene chromosomes allow cells to generate large quantities of RNA, and are found primarily in the salivary glands of Diptera and a few other animal species (Clements, 2000). However, they can also occur in germline cells in some species, making them relevant for homing gene drives. Mosquitoes are peculiar because polytene chromosomes have been observed not only in the salivary glands, but also in other tissues. The occurrence of polytene chromosomes in various organs differs between mosquito genera and species; in *Aedes aegypti* they were found only in the malphigian tubules (the main excretory organ of many insects) of adults, and the alimentary organs of the larvae (Gillham, 1957), whereas they have been identified also in the adult nurse cells of several *Anopheles* species, including *Anopheles gambiae* and *Anopheles stephensi* (Clements, 2000; George et al., 2020). The polytene chromosomes allow the nurse cells to synthesize large amounts of RNA that are to be deposited into the developing oocyte (Clements, 2000). *Drosophila* nurse cells do not contain polytene chromosomes, although their nurse cells are polyploid due to several cycles of DNA endoreplication (King et al., 1981).

The arrangement of chromosomes into polytene chromosomes may lead to more intra-chromosomal arrangements, especially for the X chromosome, which is densely packed in terms of volume (George et al., 2020). The arrangement of polytene chromosomes in nurse cells of *Anopheles* might also lead to more interactions between different chromosomes, as they are all densely packed together, even filling up the middle space of the nucleus that is usually left empty in salivary gland cells (George et al., 2020). This difference in inter-chromosomal contacts may explain why ovaries show more tissue-specific gene expression than salivary glands, as closer contact between the chromosomes could benefit co-localisation of expressed genes and the formation of transcription factories, physical areas where there are many RNA polymerases and there is high transcriptional activity (George et al., 2020).

Polytene chromosomes may be linked to the high homing rates observed in *Anopheles* (Adolfi et al., 2020; Carballar-Lejarazú et al., 2023). As is described in more detail below,

during oogenesis in females, oocytes arrests during meiosis in prophase I, which is the key moment for homing to occur. This arrest, in Diptera, is followed by oocyte maturation, during which nurse cells deposit all their contents into the oocyte before dying off. Perhaps *Anopheles* are highly efficient at producing and depositing Cas9 and gRNAs due to their polytene chromosomes at this stage, leading to more efficient Cas9 activity. Unfortunately, it may be difficult to verify if polytene chromosomes are indeed the cause behind high homing rates in *Anopheles*. In *Drosophila*, there are single-gene mutants that form polytene chromosomes in nurse cells (King et al., 1981), but these flies are sterile so cannot be tested for gene drive efficiency. Perhaps precisely measuring the amount of Cas9/gRNA protein complexes in meiotic cells at different developmental stages between species when using identical promoters, for instance via immunofluorescence staining like Weitzel et al. (2021) did for mice, could yield insight into the potential effect of polytene chromosomes. In *Drosophila* and to some extent mosquitoes, the structure of the ovaries and testes could be leveraged for this kind of analysis, as discussed in more detail below. If confirmed, this phenomenon could represent a fundamental biological constraint limiting the effectiveness of gene drives across a wide range of species.

4.6.3 Gametogenesis

Gametogenesis is assumed to be the most important stage of the germline for gene drive efficiency, since, as mentioned, meiosis I is the key moment for homing to occur. First of all, there are remarkable differences in the timing of meiosis between our species and between the sexes of each species. Second, the presence of germline stem cells could be relevant because if gene drive activity occurs there, similar to in PGCs, the outcome (homing or EJ) will remain the same for each division afterward. If EJ after gene drive activity would lead to non-viable genotypes (J. Champer, Yang, et al., 2020), this could also lead to cell apoptosis and a reduction in fertility, unless a GSC can be replaced (Spradling et al., 2011).

Across animal species, males usually have germline stem cells and actively produce spermatocytes throughout life, whereas adult females usually no longer have germline stem cells and instead store their oocytes in meiotic arrest for long periods of time (Figure 4.4A) (Saitou & Yamaji, 2012; Spradling et al., 2011). *Drosophila* is one of the few animals in which adult females do have germline stem cells (Spradling et al., 2011). *Anopheles* could also be considered an exception because almost all sperm production happens in the pupal stage

instead of during the adult stage (Clements, 2000). In mice and other mammals, all oogenesis happens early on in development, after which the oocytes are stored. Until recently, it was generally accepted that adult mammalian females did not have GSCs, though this is now being debated, with some researchers finding evidence for so called 'oogonial stem cells' that create oocytes later in life, while other methods have failed to detect stem cells in adult ovaries (Hainaut & Clarke, 2021; J. Wu et al., 2013). For each species, we compare and discuss oogenesis in females (Figure 4.5) and separately spermatogenesis in males (Figure 4.6).

Oogenesis in Drosophila

The female reproductive system of *Drosophila* contains two ovaries each composed of around 20 ovarioles (Avilés-Pagán & Orr-Weaver, 2018; Bloch Qazi et al., 2003). An ovariole could be considered an assembly line for oocytes, with the germline stem cells at the top and the most developed oocyte at the bottom (Klowden, 2007) (Figure 4.5A). There are 2-3 germline stem cells located in a niche created by somatic cells that prevents the stem cell from differentiating (Figure 4.5A, magnified box) (Fuller & Spradling, 2007). The stem cells undergo asymmetric division, and the daughter cell that is farthest from the terminal filament and cap cells differentiates. This cell will move down the ovariole and form a ball of connected cells through mitotic divisions, of which one cell will become the oocyte and initiate meiosis, whereas the others will become nurse cells that support the oocyte. The maternal proteins and mRNAs that are important for development are deposited into the oocyte by the nurse cells through the ring canals that connect them.

As mentioned above, pairing of homologous chromosomes occurs during the mitotic divisions of both female and male germline cells in Dipterans. Once GSCs differentiate, their homologous chromosomes start to pair. The chromosome alignment increases during the mitotic divisions that precede meiosis so that, by the time the cell enters meiosis, its chromosomes are already fully aligned (Joyce et al., 2013; Rong & Golic, 2003). Somatic pairing could create a longer time frame in the germline during which homologous chromosomes are close together and during which homing could occur (Figure 4.4B). This may be one of the reasons why dipterans show generally higher rates of homing across gene drive experiments compared to mammals, in which there is no mitotic pairing (McKee, 2004) and EJ is the preferred repair mechanism of DSBs in most cell types (H. Yang et al., 2020).

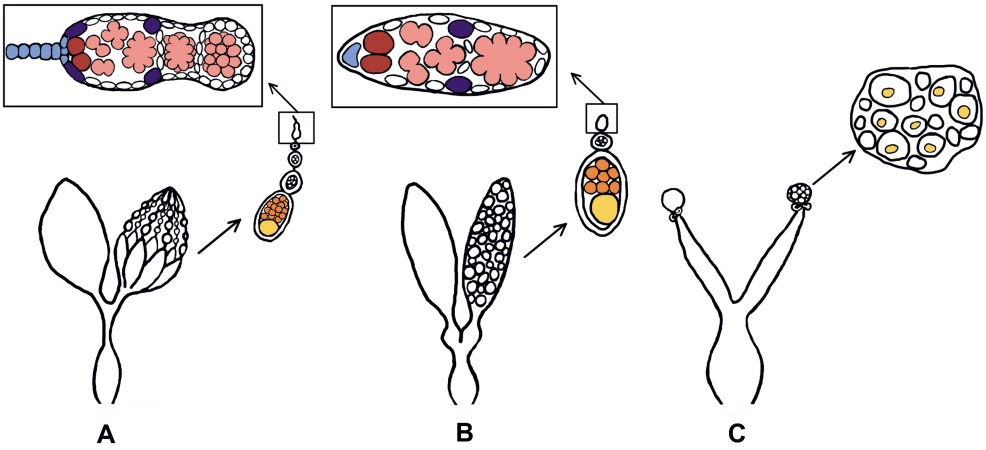


Figure 4.5. Structural overview of the ovaries of A) *Drosophila*, B) *Anopheles*, and C) mice. Close-ups show the oocytes (yellow), nurse cells (orange), germline stem cells (red), stem cell niche (light blue), cysts (pink), and somatic stem cells (dark blue). *Drosophila* ovaries are neatly arranged into ovarioles, with each ovariole containing a germarium with germline stem cells. *Anopheles* ovaries are filled with many follicles, each also containing a germarium. Mice adult ovaries do not have germline stem cells, just follicles containing oocytes. Drawings are based on Pinto (2009) (A), Foster and Walker (2019) (B), and Huang (2009) and Whary et al. (2015) (C).

The oocyte goes into meiotic arrest twice (Figure 4.4B). The first arrest is in prophase I, and is followed by oocyte maturation, during which nurse cells deposit all their contents into the oocyte before dying off (Hughes et al., 2018). This arrest during prophase I could be beneficial for gene drive homing in females (in *Drosophila*, but also mosquitoes and mice), because there is a longer window for homing to occur while chromosomes are paired. However, the oocyte is also transcriptionally silenced to a large, but not complete, extent (Navarro-Costa et al., 2016), so without fresh expression of gene drive components, the longer time-frame might not matter after a certain point if Cas9/gRNA is decaying. After the oocyte has matured, it progresses to metaphase I of meiosis and arrests again. The oocytes are stored in this second arrest until ovulation, which can happen spontaneously or in response to mating (Bloch Qazi et al., 2003). During metaphase I, the homologous chromosomes are still close together and aligned before their separation in the following phase of meiosis. It is unclear whether HDR and homing could still occur during metaphase I;

though the homologous chromosomes are still in close proximity, there may be other repair mechanisms favoured at this phase of meiosis, such as EJ, or even the accidental segregation of the fragments created by DSBs (Clay & Fox, 2021). After the subsequent meiotic divisions, three cells will form a polar body, whereas only one is fertilised and becomes the embryo (Fellmeth & McKim, 2022). Interestingly, this asymmetry in meiosis is the key evolutionary basis for a type of natural gene drive called 'centromere drive' (Malik, 2009), but this is likely not relevant for a homing gene drive as it is based on centromere binding strength, not DNA repair.

Oogenesis in mosquitoes

The process of oogenesis in mosquitoes is very similar to that in *Drosophila* and other insects, though they lack the neat organisation into ovarioles found in *Drosophila* (Figure 4.5B) (Clements, 2000). Instead, the ovaries contain many follicles, each consisting of one to three developing oocytes and a germarium, which contains the germline stem cells (Figure 4.5B, magnified box). Similar to *Drosophila*, mosquito chromosomes are also already paired up before meiosis (Mukherjee & Rees, 1970), though it remains unclear whether or not their germline stem cells and PGCs have homologous pairing too. Also, oocytes are accompanied by several nurse cells that produce mRNAs. As discussed above, during the primary meiotic arrest in prophase I in *Anopheles*, nurse cells repeatedly replicate their chromosome to form polytene chromosomes, which potentially aids gene drive activity. *Aedes* mosquitoes and *Drosophila* do not form polytene chromosomes in this tissue, which could explain their lower gene drive efficiency. Mosquitoes develop oocytes in cycles, and the follicles develop synchronously, so that at the end of each cycle, many oocytes mature simultaneously. The timing of this cycle is linked to the consumption of blood by the female. After each blood meal, one batch of eggs is laid, while the other oocyte in the follicle develops to the second resting stage, and a new oocyte is formed from the germarium. The first oocytes to be developed have started meiosis already before adult females have emerged from their pupae (Figure 4.4A). Meiotic crossing over occurs in early pachytene in prophase I, which is the stage these oocytes are in around the time mosquitoes emerge as an adult. The first oocytes of newly emerged adults continue meiosis until they are arrested in diplotene, this arrest lasts until the female feeds on blood. After blood feeding the oocytes mature and arrest again, this time in metaphase I. This arrest lasts until ovulation and fertilisation (Clements, 2000).

Perhaps an interesting way to find out if the meiotic arrest during prophase I in females makes gene drive homing more likely over time is to allow drive carriers to age before reproduction. For *Drosophila*, this would likely not matter as females will soon lay unfertilised eggs if they remain unmated (Wyman, 1979), but in mosquitoes, withholding a blood meal could prolong the time that oocytes are held in meiotic arrest. For the first batch of eggs, this arrest occurs during the diplotene stage, which is likely too late for HDR as crossing over has finished and EJ is thought to be the dominant repair mechanism during this stage (Enguita-Marruedo et al., 2019). However, for the second batch of eggs, their preceding meiotic cells have rested in an earlier stage of Prophase I and could show an effect of ageing in the form of higher homing rates. Besides teasing apart developmental stages relevant for gene drive functioning, the effect of ageing could matter in an ecological setting if mosquitoes are able to lay multiple batches of eggs.

Oogenesis in mice

Soon after settling in the gonads, the PGCs in female mice embryos start differentiating and enter meiosis, in response to signals from nearby somatic cells (Reik & Surani, 2015). The early oocytes undergo meiotic crossing over and then arrest in the diplotene stage of prophase I. Adult mammalian females have a menstrual cycle in which a peak in hormones leads to resumption of meiosis in an oocyte, after which they are arrested in metaphase II until fertilisation (Figure 4.5C) (Sha et al., 2019).

In contrast to *Drosophila* and mosquitoes, mice only pair up homologous chromosomes during prophase I of meiosis. Cells undergoing mitosis do not appear to repair DSBs that are created after late prophase with HDR (Hustedt & Durocher, 2017). This difference points towards the importance of the timing of nuclease expression, which likely requires greater precision in mice than Dipterans. As mentioned before, Pfitzner et al. (2020) found that *vasa* is likely expressed too little and too early in the germline, and no homing was detected in females or males. When Grunwald et al. (2019) used a *vasa*-Cre system (where Cas9 was expressed under a strong constitutive promoter, but surrounded by *loxP* sites that stop expression, until the *loxP* sites were removed by the Cre protein, driven by the *vasa* promoter), which increases as well as delays Cas9 expression compared to direct *vasa* expression, some homing was observed in female mice, although results were highly variable between individuals. One reason for this low efficiency could be that the expression

level of the *vasa* homolog in mice is quite different from *Anopheles* and *Drosophila*, as it seems to be expressed in many somatic tissues (Baldarelli et al., 2024), as well as involved in PGC proliferation and spermatogenesis in males specifically (Tanaka et al., 2000). Interestingly, research in knockouts of the gene suggests that it is not essential to the development of germ cells in females (Tanaka et al., 2000). This is in contrast to the function of *vasa* in *Drosophila*, where it is not essential for spermatogenesis, but is required especially for oogenesis (Lasko & Ashburner, 1990; Renault, 2012; Styhler et al., 1998; Tomancak et al., 1998). Where *vasa* in *Drosophila* has higher expression in females than males, the opposite is true for mice (Pfitzner et al., 2020). Additionally, Grunwald et al. (2019) observed that in mice, many more chromosomes remained uncleaved by Cas9 in females than in males, where this never occurred. Thus, if in female mice higher nuclease expression can be achieved with the same temporal expression pattern, higher homing rates will likely result. Other promoters besides *vasa* could be harnessed for this purpose, or perhaps synthetic elements can be added to *vasa* or another promoter to tweak nuclease expression expression levels and timing. Similar to mosquitoes, investigating whether ageing influences gene drive efficiency in mice could indicate whether the meiotic arrest during prophase I is relevant for homing efficiency, although the arrest is in the diplotene stage, which is likely too late for HDR to occur.

Spermatogenesis in Drosophila

In *Drosophila* males, germline stem cells are maintained in a niche at the tip of the testes (Figure 4.6A) (Fuller & Spradling, 2007). This niche contains between 6-12 germline stem cells, which each undergo several incomplete mitotic divisions to form 16 connected spermatogonia. The spermatogonia undergo meiosis to first become spermatocytes and eventually spermatids. Spermatids then undergo spermiogenesis, a developmental process involving DNA condensation, the development of microtubules, and elongation of the flagellum, after which the spermatids have become spermatozoa. Besides germline stem cells, the tip of the testes also contains somatic stem cells that produce somatic cells to enclose the dividing germline cells, as well as a hub of cells that form the stem cell niche and maintain stem cell populations (Klowden, 2007).

Similar to *Drosophila* females, GSCs first undergo four mitotic divisions before meiosis starts. As opposed to female meiosis, where only one final meiotic cell becomes an oocyte,

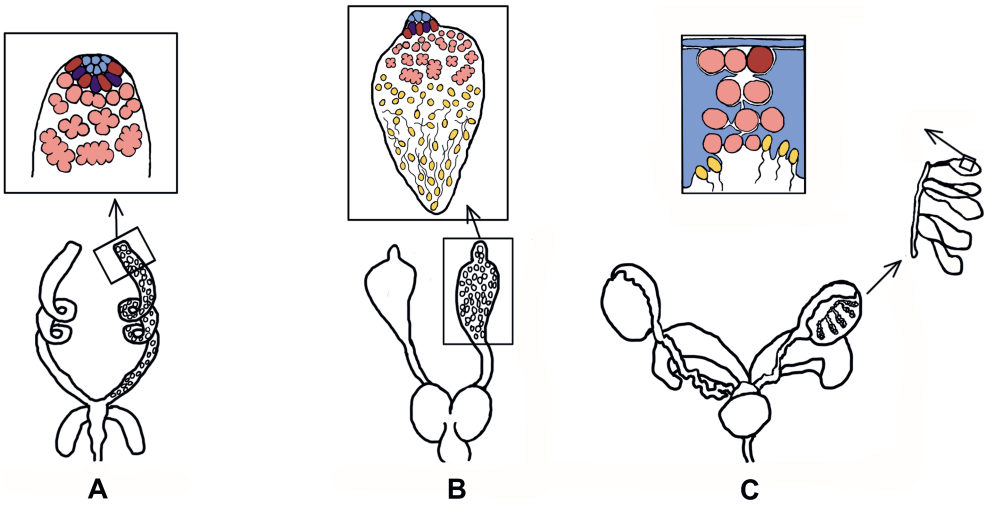


Figure 4.6. Structural overview of the testes of A) *Drosophila*, B) *Anopheles*, and C) mice. Close-ups show the germline stem cells (red), stem cell niche (light blue), the intermediate stages between germline stem cell and spermatids (orange), spermatids (yellow) and somatic stem cells (dark blue). Drawings are based on Greenspan and Matunis (2023) and L. Li and Xie (2005) (A), Vitale et al. (2023) (B), and Whary et al. (2015) and L. Li and Xie (2005) (C).

all four final meiotic cells in males become sperm cells. Meiosis in male *Drosophila* is peculiar in several ways. First of all, meiosis is achiasmatic, meaning that there is no crossing over between homologous chromosomes in meiosis I. Homologous chromosomes are aligned during prophase I, but chromosomes are not condensed at this time like they are in females (McKee et al., 2012). Next, chromosomes quickly condense and lose their homologous pairing, followed by centromere pairing before segregation. It is not entirely understood how chromosomes remain connected, but evidence suggests that *Drosophila* males rely on specific loci on the chromosomes (Lee & Rosin, 2024) and some evolutionarily novel proteins for this purpose (McKee et al., 2012). If one were to design a gene drive that is only active in males, with the purpose of avoiding embryo activity after maternal deposition, genes involved in male meiosis could be promising candidates for Cas9 promoters. How this atypical meiotic program influences gene drive efficiency is not known, but possibly uncondensed chromatin in combination with early homologous pairing makes the DNA more accessible for Cas9 activity and HDR. Indeed, Terradas et al. (2021) have suggested

that their observed reduced homing efficiency in *Drosophila* males might be linked to their lack of homologous recombination during meiosis, as this effect seemed consistent across different constructs. However, other studies have found no indication of consistent homing efficiency differences between female and male *Drosophila*, so it is unclear to what extent this could be true (Du et al., 2024). Interestingly, females of some Lepidopteran species also have achiasmatic meiosis (Xiang et al., 2023). However, one of the few gene drive studies in a non-Dipteran insect, namely the diamondback moth, showed no detectable homing in either males or females (total cleavage rates were also minimal), so it was impossible to assess what role achiasmatic meiosis played (X. Xu et al., 2022).

Spermatogenesis in mosquitoes

In mosquitoes, most spermatogenesis occurs during the larval and pupal stages of the life cycle, rather than in the adult (Figure 4.4A). The production of new sperm cells may continue in adults of some species, such as *Aedes aegypti*, but this is negligible compared to the amounts of sperm produced earlier on. The testes are organised into layers; each layer contains germ cells in a different developmental stage, and the most developed stages are located in the posterior region (Figure 4.6B). The mitotic and meiotic divisions of germline stem cells into eventual spermatozoa is the same as described for *Drosophila*, though little is known about the first step, in which GSCs form spermatogonia. When the cells enter meiosis, the homologous chromosomes are already paired due to mitotic pairing, so there is no need for the pairing stages of prophase I, as there is a gradual transition from mitotic pairing to meiotic pairing of homologous chromosomes (Clements, 2000).

Unfortunately, meiosis in mosquitoes is not as well-studied as in *Drosophila*, so it is unclear why homing gene drives could be so much more efficient in *Anopheles* mosquitoes than in *Aedes* or *Drosophila*. As mentioned, in male mosquitoes, most sperm production happens in the pupal stage, so a gene drive would need to be expressed at this time. In contrast, *Drosophila* and mice produce sperm throughout their lives, meaning Cas9 needs to be active consistently for homing efficiency to remain high. Achiasmatic meiosis might offer a potential biological explanation for differences in homing between the two insects, as mosquito males do form chiasmata during meiosis where *Drosophila* males do not (Clements, 2000). However, similar to in *Drosophila*, drive efficiency seems more promoter-specific rather than sex-specific in mosquitoes (M. A. E. Anderson et al., 2023),

so it seems unlikely that male-specific meiotic processes are highly influential. Therefore, for *Aedes* mosquitoes, the identification of alternative Cas9 promoters with appropriate expression patterns in both males and females seems to be the most promising strategy for gene drive optimisation (M. A. E. Anderson et al., 2023). Single cell sequencing technology could be leveraged, similar to studies in *Anopheles* (Page et al., 2023).

Spermatogenesis in mice

In mice, sperm production happens throughout life. Upon birth, germ cells are arrested in mitosis (Reik & Surani, 2015). They enter meiosis about eight days later and begin production of the first batch of sperm cells. Meiosis lasts around 14 days, so the first round of meiosis starts in spermatogonia at day 8 and ends in spermatids around day 22. The pachytene stage, the phase of prophase I that is associated with crossing over, initiates around days 12 to 14 (Margolin et al., 2014). Later batches of sperm cells are produced less synchronously across the testes.

In male mice, a promoter is needed that is germline specific, strongly expressed, and expressed right before or during early prophase I of meiosis. Only one study has reported any homing in male mice so far (Weitzel et al., 2021), where the strategy to achieve homing in male mice was to select a Cas9 promoter that is directly involved in meiosis during prophase I. Such promoters likely circumvent the issue of differential expression between females and males, which for the *vasa* promoter was shown to be problematic (Figure 4.4A) (Grunwald et al., 2019, 2022). Weitzel et al. (2021) inserted their Cas9 construct directly after Spo11, a gene involved in double strand break formation for crossing-over (Oger & Claeys Bouuaert, 2025; Romanienko & Camerini-Otero, 2000; Z. Zheng et al., 2025), so they are expressed in conjunction. This way, Cas9 should be endogenously expressed at the right interval for meiosis in both males and females. A gene drive conversion rate of 11% was achieved in male mice, which is a promising finding. However, overall Cas9 expression was again too low in both sexes as very few DSBs were formed altogether. These results underscore the importance of both the timing and the level of Cas9 expression for gene drive homing in mice.

An alternative gene that we suggest might have a favourable expression pattern in mice is Prdm9, which is responsible for targeting Spo11 to specific genomic locations for subsequent double strand break formation, including creating the required open chromatin

state during prophase I (Grey et al., 2018). With this promoter, Cas9 might be expressed early enough to reach sufficient levels, and still be active at the right time for HDR to take place. Another tactic could be to look at single cell RNA sequencing data of the ovaries and testes at the time of meiosis to find genes that are expressed in the right time frame in both sexes. J.-J. Wang et al. (2020) looked at single cell RNA sequencing data of ovaries over time and identified genes active in different stages. Similarly, Margolin et al. (2014) looked at RNA sequencing data of the whole testes of mice at several different developmental time-points to find genes involved in meiosis. With this data, genes can be found that are highly expressed at the right time during early meiosis. Both studies, however, only showed data from the stages around meiosis and later developmental stages, so these studies cannot say whether these genes are also expressed in earlier stages of the germline and somatic cells, which is undesirable.

A final interesting phenomenon during spermatogenesis in mice is the extensive remodelling of the chromatin structure during spermatogenesis, which leads to gene expression repression as well as chromosome pairing (Reik & Surani, 2015; Sasaki & Matsui, 2008). Once the homologous chromosomes are paired and enter the pachytene stage of prophase I, the X and Y chromosomes are silenced, and they remain repressed until the end of spermatogenesis (Turner, 2007). This phenomenon is called meiotic sex chromosome inactivation, and in mammals, it only occurs in the male during spermatogenesis (Turner, 2007). In insects, less is known about meiotic sex chromosome inactivation, though there is evidence suggesting it occurs in *Drosophila* males as well (Kaiser & Bachtrog, 2010). Any gene drive that is expressed from a sex-chromosome would thus be silenced too. However, homing gene drives are usually not designed this way, as homing could only occur in females, and the gene drive may impose its fitness cost directly in hemizygous males. An interesting phenomenon that may be tangentially relevant for homing gene drives was observed in Bunting et al. (2024), who built an X-shredder system (which can be a driving Y gene drive when located on the Y chromosome). An X-shredder gene drive targets the X-chromosome at several loci, shredding the chromosome beyond repair and making X-bearing sperm non-viable. In this study (where the gene drive was expressed from several random integration sites in the genome), no bias towards males was observed, only a reduction in fertility in males (Bunting et al., 2024). This lower fertility was due to non-viable sperm blocking spermatogenesis, likely due to the denser cellular structure in mouse testes (Figure 4.6C). Likely, any homing gene drive that would lead to an increase of non-viable

sperm would run into this developmental blockade.

4.7 Conclusion

In many species in which it has been tested, gene drive technology has not yet achieved the efficiency needed for consideration for practical use (Verkuijl, 2023). Moreover, it is not known why gene drives work well in some species but not in others (Ang et al., 2025). In this perspective article, we outline how the gene drive field may have been operating under an overly simplistic view of the germline environment in which a homing gene drive is active, and arguably more importantly, have been ignoring key differences in the developmental biology of their focal species (Table 4.1). Yet, we argue that exactly these differences may form the basis for understanding the reasons for the between-species differences in gene drive efficiency. Importantly, in our opinion, this understanding provides ample opportunities to species-specifically improve gene drive efficiency. Therefore, with this paper, we hope to encourage collaboration between gene drive biotechnologists, working with a single or various species, and developmental biologists to promote interdisciplinary research that may prove essential for further advances of gene drive technology. Collaboration may become increasingly important as the field progresses to testing gene drives in non-model organisms that are likely to have different or under-researched germline biologies (X. Xu et al., 2022). Even though it is not always essential for a homing gene drive to be 100% efficient (Faber et al., 2024), it is still desirable, especially in the face of evolving resistance (Faber, Champer, et al., 2025; Price et al., 2020).

Gene drives are complex constructs active during intricate processes, and their efficiency is dependent on many factors. For homing to occur, the nuclease needs to be expressed at the right time in the right place and in sufficiently high levels. In addition, the target gene needs to be accessible, and preferably highly conserved to avoid resistance from forming. Even then, there might be further complicating cell biological factors in the germline such as somatic pairing of chromosomes, polytene chromosomes, achiasmatic meiosis, and differential timing of meiosis between males and females (Table 4.1). Even if homing was successful and the expression of the nuclease was limited to the germline, there can still be problems from maternally deposited nucleases. These different factors affect dipterans and mice in distinct ways.

Table 4.1. Developmental processes and their suspected effect on homing gene drive efficiency: beneficial (+), detrimental (–), or unknown (?), and if they are relevant to different species.

Developmental process	Effect on gene drive efficiency	Relevant for species			
		Drosophila	*Anopheles* spp.	*Aedes* spp.	Mice
Deposition of maternal factors	–	x	x	x	
Posterior localisation of germline-specifying factors	+?	x	x?	x?	
Syncytial early development	–?	x			
Early zygotic genome activation	+?				x
Germline-specific chromatin inaccessibility	–?				x?
Tissue-specific cis-regulatory elements	–?	x	x	x	x?
PGC and GSC gene expression	–	x	x	x	x
Somatic/mitotic pairing of chromosomes	+	x	x	x	
Polytene chromosomes in nurse cells	+?		x	?	
Sex-differential timing of meiosis	–?	x			x
Presence of GSCs	?	♀ & ♂	♂	♂	♂
Transcriptional silencing of gametocytes	–?	x	x	x	x
Meiotic I arrest	?	♀	♀	♀	♀
Achiasmatic meiosis	–?	x			

Although these biological differences might set an upper limit to the efficiency a homing gene drive can achieve in some species discussed here, considerable improvements have been made by testing a range of different Cas9 promoters for germline specific genes that are highly expressed, yet that also avoid maternal deposition of Cas9 in Dipterans (M. A. E. Anderson et al., 2023; Du et al., 2024; Weitzel et al., 2021; Y. Wu et al., 2025). The most important factor for choosing new promoter candidates going forward is to use expression data from the species itself, as opposed to homology between species, and with as much temporal resolution as possible. We must not forget that although molecular processes such as meiosis may seem quite conserved across the tree of life, species can still be vastly different in subtle ways. Differential timing of development and its associated gene expression between species is called heterochrony (Keyte & Smith, 2014), a phenomenon that is highly relevant for germlines and gene drives. Although the nuclease promoter is likely still the most important factor to optimise going forward, further improvements might be possible by altering other aspects of the gene drive construct, such as which gene was targeted and where it is located, or adding other synthetic components to the gene drive to tweak expression. Overall, we believe that a perspective firmly based in developmental biology will be essential to unlock the full potential of gene drive technology, so it can contribute to sustainable invasive pest control in a wide range of species in the future.

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Author contributions

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"His brain warns him that there are words that cover up the world. There are words that are convenient, hygienic. Legal."

— Agustina Bazterrica, Tender Is the Flesh

"There is no such thing as a good influence. Because to influence a person is to give him one's own soul. He does not think his natural thoughts, or burn with his natural passions. His virtues are not real to him. His sins, if there are such thing as sins, are borrowed. He becomes an echo of someone else's music, an actor of a part that has not been written for him."

— Oscar Wilde, The Picture of Dorian Gray

Chapter 5

Improving the suppressive power of homing gene drive by co-targeting a distant-site female fertility gene

This chapter is based on:

Faber, N. R.*, Xu, X.*, Chen, J., Hou, S., Du, J., Pannebakker, B. A., Zwaan, B. J., Van Den Heuvel, J., & Champer, J. (2024). Improving the suppressive power of homing gene drive by co-targeting a distant-site female fertility gene. *Nature Communications, 15*(1), 9249. https://doi.org/10.1038/s41467-024-53631-5

*Equal contribution

5.1 Abstract

Gene drive technology has the potential to address major biological challenges. Well-studied homing suppression drives have been shown to be highly efficient in *Anopheles* mosquitoes, but for other organisms, lower rates of drive conversion prevent elimination of the target population. To tackle this issue, we propose a gene drive design that has two targets: a drive homing site where drive conversion takes place, and a distant site where cleavage induces population suppression. We model this design and find that the two-target system allows suppression to occur over a much wider range of drive conversion efficiency. Specifically, the cutting efficiency now determines the suppressive power of the drive, rather than the conversion efficiency as in standard suppression drives. We construct a two-target drive in *Drosophila melanogaster* and show that both components of the gene drive function successfully. However, cleavage in the embryo from maternal deposition as well as fitness costs in female drive heterozygotes both remain significant challenges for both two-target and standard suppression drives. Overall, our improved gene drive design has the potential to ease problems associated with homing suppression gene drives for many species where drive conversion is less efficient.

5.2 Introduction

Gene drive technology could be a valuable tool to help address major challenges posed by populations of pest species. These populations include disease vectors, invasive species, and agricultural pests, for which often no effective method of control is available (Bier, 2022; J. Champer et al., 2016; Esvelt et al., 2014; Godfray et al., 2017; G.-H. Wang et al., 2022). A gene drive is a genetic element that biases its inheritance in its own favor, allowing it to spread through a population over generations (Alphey et al., 2020). Synthetic gene drives can be designed for population modification, for example to immunise a population of mosquitoes against malaria parasites (Gantz et al., 2015), or for population suppression, for example to eliminate a population of mosquitoes (Kyrou et al., 2018) or invasive pests (Gierus et al., 2022; Prowse et al., 2017). Released in the target population, suppression gene drives are designed to spread at a rapid rate and carry a recessive fitness cost, thus causing a decline in population size or even complete elimination (S. E. Champer et al., 2021; Kyrou et al., 2018; Wilkins et al., 2018). Suppression gene drives have benefits over conventional methods of control because they are species-specific (and thus more ecologically friendly), as well as potentially more efficient and more humane, though there are challenges regarding localisation and containment for some drive types (Esvelt & Gemmell, 2017).

There are many different types of gene drive and what distinguishes them the most is how they handle the trade-off between efficiency of spread and confinement (Alphey et al., 2020; J. Champer et al., 2016; G.-H. Wang et al., 2022). The most efficient and well-studied type of suppression drive is the CRISPR-Cas9-based homing drive. In drive heterozygotes, the gene drive copies itself to the homologous chromosome in the germline through a process called "homing" or "drive conversion". The gene drive incurs a recessive fitness cost by being located inside a haplosufficient female fertility gene, so drive homozygous females are sterile. Ideally, the female infertility is completely recessive, so that there are no fitness costs for drive heterozygotes. As the frequency of the gene drive increases, more sterile female offspring are created, thus imposing a genetic load on the population (Dhole et al., 2020). If the frequency of sterile females in the population is sufficiently high, the population can be completely eliminated (J. Champer et al., 2016; Prowse et al., 2017).

Although homing gene drives are very promising in theory, *in vivo* tests in several organisms

have revealed practical challenges, so complete population suppression is not yet attainable in most species (Figure 5.1) (J. Champer et al., 2017; S. E. Champer et al., 2020; Esvelt & Gemmell, 2017; Grunwald et al., 2019; Hammond et al., 2017; Pfitzner et al., 2020; Price et al., 2020; Prowse et al., 2017). Homing suppression gene drives face two major challenges, the first being formation of functional resistance alleles (Carrami et al., 2018; J. Champer et al., 2017; Hammond et al., 2017; Pham et al., 2019). A typical gene drive is active in the germline, and the primary way in which Cas9 cuts will be repaired is through homology-directed repair (Rode et al., 2019). In this pathway, the cell uses the drive chromosome as a template, copying its sequence to the other chromosome. Sometimes, particularly if the cut is made outside of the time window in which this repair pathway is preferred, repair can occur via end-joining instead. This repair pathway is error-prone and often leads to small insertions and/or deletions. Because of these mutations, the gene drive guide RNA (gRNA) usually cannot recognise the new sequence, making it a resistance allele that can no longer be converted to a drive allele. Resistance alleles that preserve the function of the target gene ("r1" alleles) are problematic as there is very strong selection for this allele which will rescue the population from the suppression drive (Hammond et al., 2017). non-functional resistance alleles ("r2" alleles), on the other hand, will not be able to rescue the population because of their deleterious nature (J. Champer et al., 2017). Although r2 alleles can reduce drive efficiency, the less common r1 alleles are the bigger problem because they will out-compete the gene drive in the population. There are several strategies to avoid the formation of r1 alleles in suppression drives, which include targeting an extremely conserved locus (Kyrou et al., 2018), multiplexing gRNAs (S. E. Champer et al., 2020; Oberhofer et al., 2018; E. Yang et al., 2022), and using improved promoters (Carballar-Lejarazú et al., 2020; Du et al., 2024; Hammond, Karlsson, et al., 2021).

Besides r1 alleles, a second challenge for suppression gene drives is low drive conversion, which is the rate at which wild type alleles are converted into drive alleles (Pfitzner et al., 2020; E. Yang et al., 2022). In *Anopheles* mosquitoes, drive conversion has been shown to reach 95-99% (Hammond et al., 2016; Kyrou et al., 2018). In *Aedes* mosquitoes and *Drosophila melanogaster* on the other hand, the conversion rate is usually significantly lower, around 50 to 70% for most constructs (J. Champer et al., 2017; M. Li et al., 2020; Reid et al., 2022b; E. Yang et al., 2022). Additionally, it appears that multiplexing gRNAs, which is a way to avoid r1 alleles, may further reduce conversion rates when using more than 2-4 gRNAs (S. E. Champer et al., 2020; E. Yang et al., 2022). This low efficiency re-

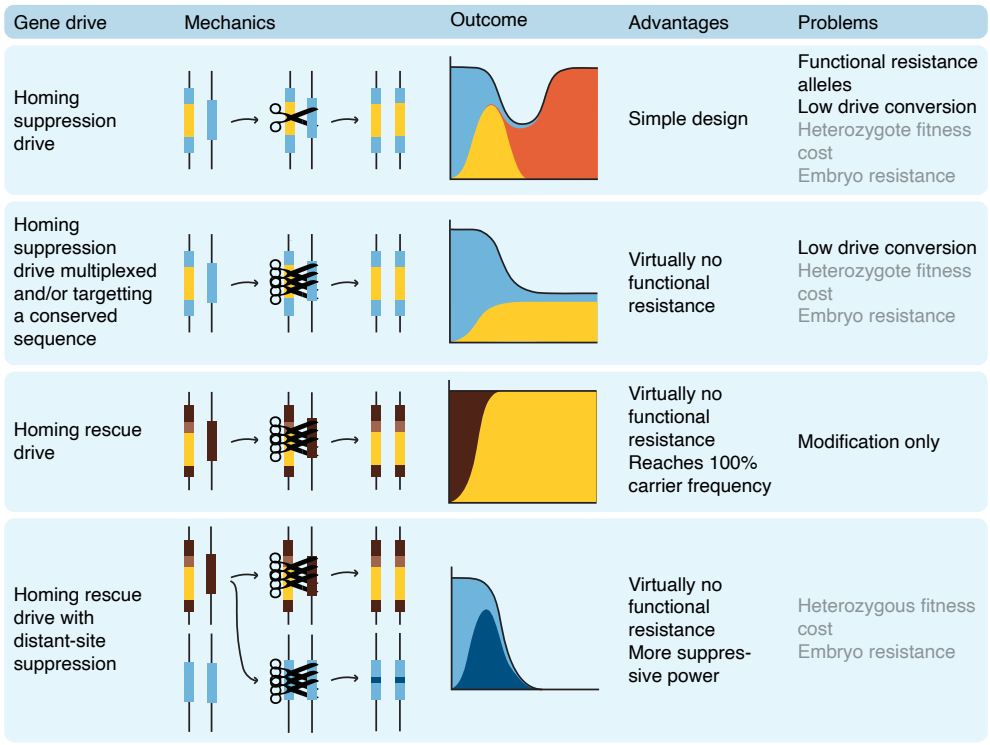


Figure 5.1. Overview of several homing gene drive designs, their advantages, and major challenges they face. In the outcomes graphs, light blue = female fertility gene, yellow = gene drive, orange/red = functional resistance allele, dark brown = essential gene, dark blue = non-functional resistance allele (only the female fertility target site is displayed for the distant-site suppression system). Additionally in the schematics, light brown = essential gene rescue. Primary problems for drive designs are in black, and secondary problems in grey.

sults in partial population suppression instead of population elimination. The amount by which the population size is reduced compared to the expected population size in the absence of the gene drive is related to the genetic load of the gene drive when its reaches its equilibrium frequency in the population (Dhole et al., 2020). The genetic load needed to eliminate a target population increases for higher low-density growth rates (the reproductive advantage individuals experience in the absence of competition). Drive conversion efficiency seems to differ between species for the most commonly used promoters, though improvements might be possible by testing additional promoters (M. A. E. Anderson et al.,

2023; Carballar-Lejarazú et al., 2022; Chan et al., 2013; Du et al., 2024; Hammond, Karlsson, et al., 2021; Pfitzner et al., 2020), or perhaps by more strongly localising the gene drive mRNA to the nucleus (Yadav et al., 2023).

To tackle this issue, we propose a gene drive design that has two targets: a homing site where drive conversion takes place, and a distant cutting site (where the drive is not present) for providing the fitness cost for population suppression (Figure 5.1). For the homing site, we harness a modification drive that is located in an essential gene (thereby disrupting it) while also providing a rescue for this gene (Figure 5.1) (J. Champer, Yang, et al., 2020). A previous study has already demonstrated the practical feasibility of using a homing gene drive while also targeting another gene for population modification (Kandul et al., 2020), and another recent study has shown that modifying a natural gene drive to also target a distant female fertility gene is feasible for population suppression (Gierus et al., 2022). Thus, we model the two-target design, comparing it to a standard homing suppression gene drive, and find that this two-target system allows suppression to occur over a much wider range of drive conversion efficiency. Most notably, the suppressive power now depends usually on cutting efficiency at the distant target instead of drive conversion, which is advantageous because the total cutting rate is easier to increase and has often been substantially higher than drive conversion rate (M. A. E. Anderson et al., 2023; J. Champer et al., 2017; Grunwald et al., 2019; Pfitzner et al., 2020). We construct this drive in *Drosophila melanogaster* and show that both components function successfully. We analyse the relevant genomic regions in which the drive components are inserted and find an unexpected sequence complexity. Nevertheless, the drive conversion rate is within the range generally observed in *D. melanogaster*, and the cut rate at the distant-site target is very high. However, high fitness costs still thwarts the drive's success in cage populations, a factor that could potentially be problematic in any suppression drive based on targeting essential genes. Nevertheless, our improved gene drive design enables development of strong homing suppression gene drives for a wide array of species where drive conversion is less efficient.

5.3 Results

In our main modeling results, we show the results of three gene drives: a standard female fertility homing suppression drive and two two-target drives that target a female fertility gene at a distant site for population suppression. These two-target drives are located in

and provide rescue for a haplolethal or a haplosufficient gene. Drive conversion occurs normally in these, but they also cut and disrupt a distant-site female fertility target without rescue. Figure 5.2 shows the three drives' components, their activity, and also the alleles and phenotypes that can result in gametes from drive activity.

5.3.1 Cut and conversion rate

Because the drive conversion rate is one of the most important parameters to determine the success of a suppression drive, we start by varying it, together with the total cut rate (referring to germline cutting) (S. E. Champer et al., 2021). Any wild-type alleles that are cut but not converted to drive alleles are converted to non-functional resistance alleles (Figure 5.3A). At the distant female fertility gene target, there is no drive allele present, so the total cut rate can only result in non-functional resistance alleles. Besides these, all other parameters are fixed at optimum values (no embryo resistance, no fitness costs, no functional resistance, and no effect of Cas9 saturation). Figure 5.3B shows population size over time after a gene drive introduction.

At 100% cutting and drive conversion, all three drives work equally well, rapidly eliminating the population. As the conversion rate decreases slightly, we still observe drive success, but stochasticity now plays an important role in achieving population elimination for the standard suppression drive. Further reducing drive conversion results first in the failure of the standard suppression drive, and then the haplosufficient rescue drive as well (though given more time it may still achieve complete suppression), whereas the haplolethal rescue drive remains successfully reliably and relatively efficient.

These major differences between drives can be explained by the standard suppression drive relying on drive conversion to both spread and suppress the population, whereas both two-target drives use two separate loci for this (Figure 5.2). The haplolethal distant-site drive can make r2 alleles at the drive locus that are immediately removed due to the haplolethal nature of the gene (Figure S5.1A). Therefore, this gene drive spreads the most efficiently. The haplosufficient rescue drive and the standard suppression drive also form deleterious r2 alleles, but these can remain in the population due to slower removal, which impairs drive spread because drive conversion will not occur in drive/r2 heterozygotes (also an increase in r2 alleles cause a mild increase in stochasticity). Additionally, in the standard suppression drive, not only do r2 alleles remain in the population and impair homing, but they also

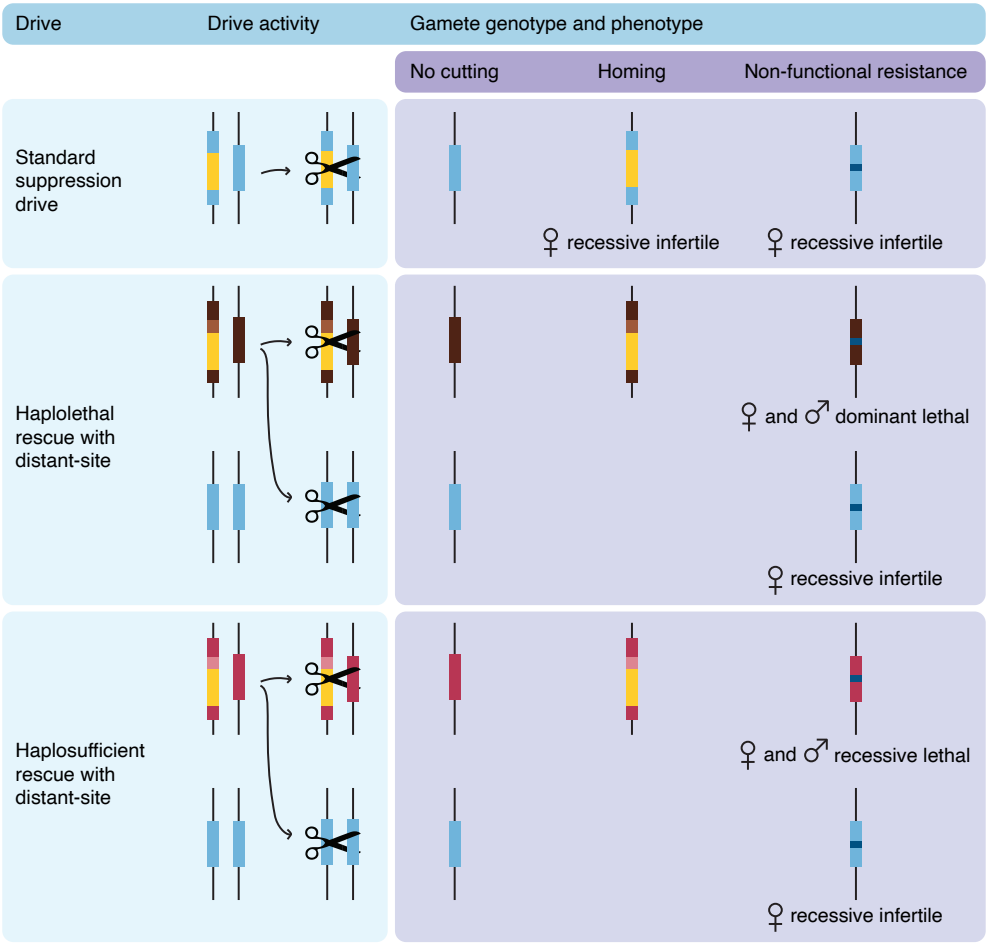


Figure 5.2. Overview of our three modelled drives, their activity, and the resulting gamete genotypes and phenotypes. We do not show functional resistance alleles, but their phenotypes would be the same as wild-type. Light blue = female fertility gene, yellow = gene drive, dark blue = non-functional resistance allele, dark brown = haplolethal essential gene, light brown = haplolethal essential gene rescue, dark pink = haplosufficient essential gene, light pink = haplosufficient essential gene rescue.

decrease the drive frequency (female drive/r2 heterozygotes are sterile) (Figure S5.1).

As the cutting rate decreases with constant drive conversion, we see that the standard suppression drive is not heavily impacted (its genetic load depends heavily on the conversion

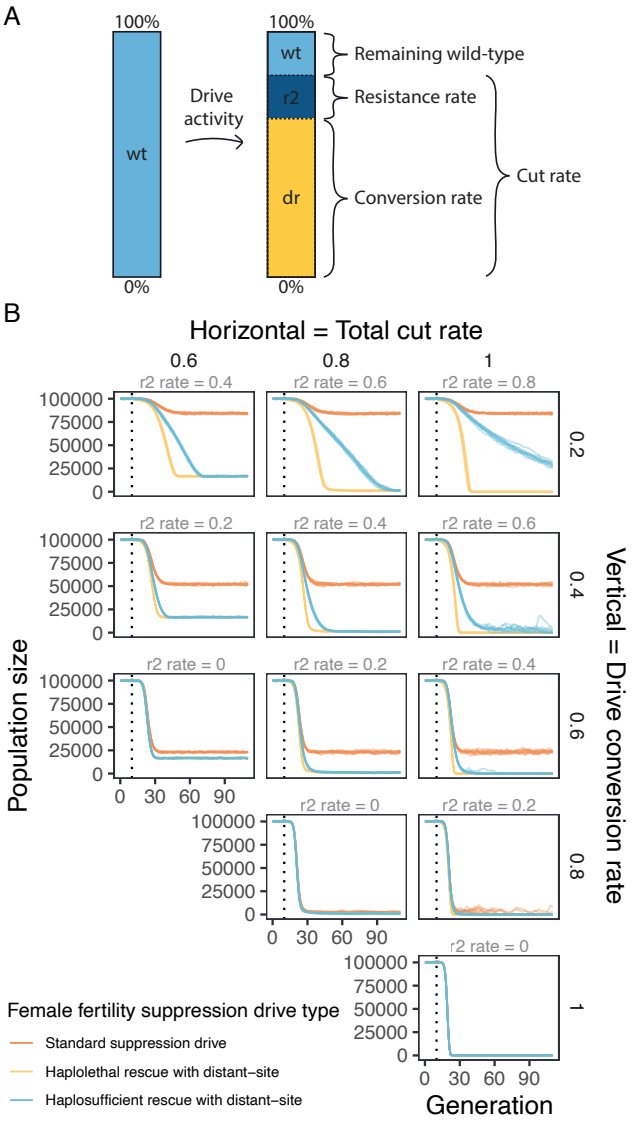


Figure 5.3. Example population trajectories under varying cut and conversion rates. A) Schematic of drive activity rates in the germline. B) Population suppression with varying total cut and drive conversion rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. Note that both drive conversion rate and total cut rate are absolute rates, so the drive conversion rate can never be higher than the total cut rate. For each combination of parameters, we ran 10 model repetitions for each drive that are each shown as a translucent line.

rate, with germline resistance alleles having little effect), whereas the distant-site suppression drives do not lose their effectiveness until the total cut rate falls significantly (Figure 5.3 and Figure S5.2). Because the distant site drives use the total cut rate for disruption of the female fertility target, this total cut rate largely determines the suppressive power rather than drive conversion. Though the distant-site haplosufficient rescue drive is slower to spread due to reduced ability to remove r2 alleles, they both eventually reach the same equilibrium population suppression (Figure S5.2).

For each gene drive, we determined the complete suppression success rate and the genetic load (Figure 5.4). The genetic load is the suppressive power of a drive, defined as the reduction in reproduction of the population compared to a wild-type population of the same size (Dhole et al., 2020). In Figure 5.4A, we see that the standard suppression drive has the smallest area of population elimination success, followed by the haplosufficient two-target drive, and then the haplolethal two-target drive. The standard suppression drive requires a high drive conversion rate to eliminate the population, whereas both two-target drives rely mostly on the cut rate alone. The same pattern is visible in the genetic load in Figure 5.4B, where the genetic load of the standard suppression drive relies on the conversion rate only, whereas both two-target drives rely almost entirely on the cut rate for their genetic load.

The haplosufficient rescue drive shows some more complex dynamics in some areas of parameter space in Figure 5.4B. When the cut rate is 1 but the drive conversion is low, the genetic load is reduced because the drive itself is not able reach high frequency due to r2 alleles are blocking its progress (Figure S5.3). The drive spreads best with the highest genetic load when the total cut rate is somewhat below 1. At the same time, high cut rates are still necessary at the distant site to achieve population complete suppression (Figure S5.3).

Another notable dynamic for both two-target drives in Figure 5.4B is the bottom row, where drive conversion is 0. Here, the two two-target drives become identical to a version of two toxin-antidote drives previously described called a TADE (Toxin Antidote Dominant Embryo) suppression drive and a TARE (Toxin Antidote Recessive Embryo) suppression drive (J. Champer, Champer, et al., 2021). These drives show density-dependent dynamics, so their ability to increase in frequency depends on their frequency as well as their total cut rate, hence the lack of suppression success for the haplolethal rescue drive due to our

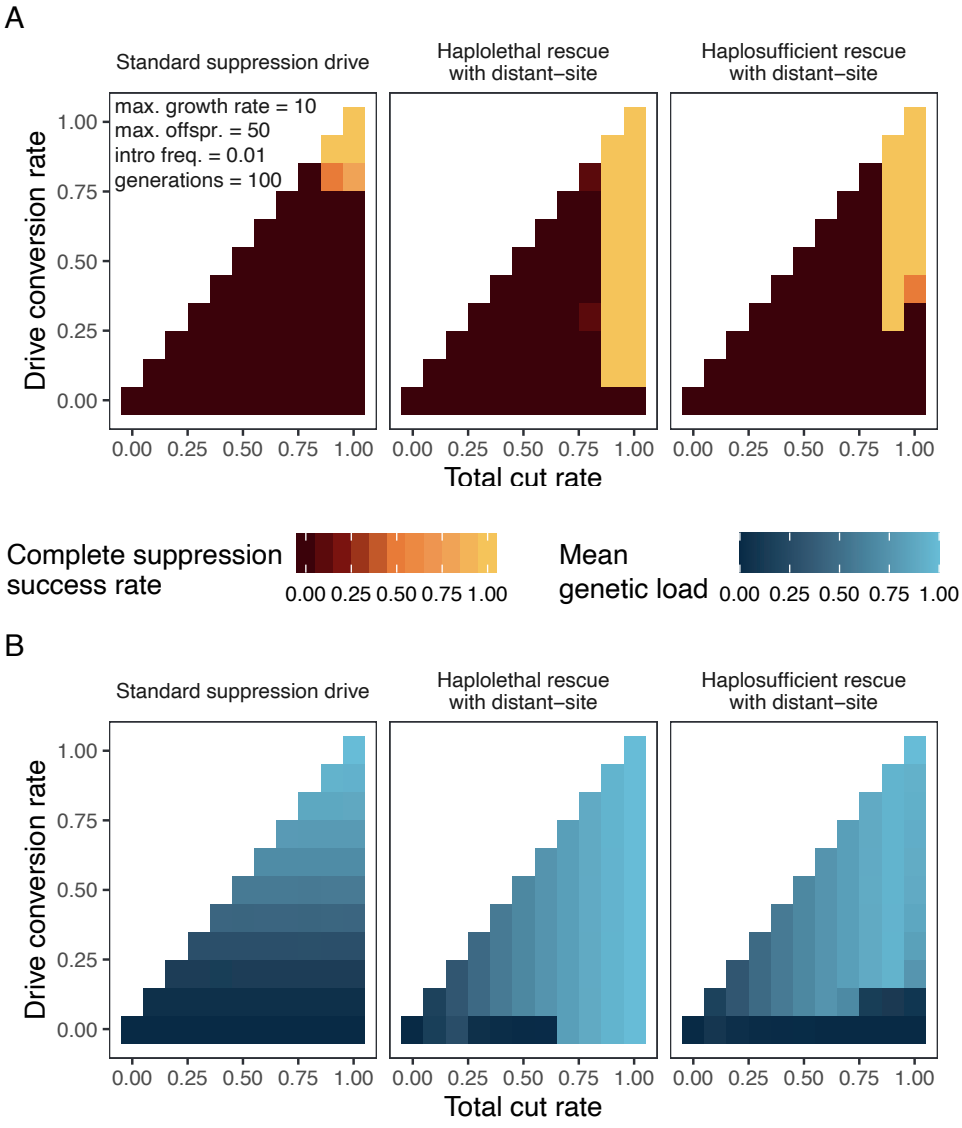


Figure 5.4. Drive performance under varying cut and conversion rates. A) Complete suppression success rate and B) mean genetic load with variable total cut and drive conversion rates. The complete suppression success rate is calculated as the number of repetitions in which population elimination occurs within 100 generations after drive introduction divided by the total number of repetitions. For each combination of parameters, we ran 10 model repetitions.

small release size. In the case of the haplolethal two-target drive, due to the additional disruption of the distant-site female fertility gene, a cut rate of at least 0.7 is necessary for the drive to increase in frequency. The TARE-like suppression drive is not able to reach a high genetic load in the first place (J. Champer, Champer, et al., 2021). Interestingly, at very low cut rates, we observe that the two-target drives are able to remain in the population long enough for the distant-site to be disrupted up to a certain frequency, after which the gene drive can sometimes induce a small genetic load during the simulation.

5.3.2 Embryo cut rate and somatic expression effects on fitness

Two other important determinants of drive success are the embryo cut rate and fitness costs in heterozygous females based on the disruption of wild-type alleles when they are needed for fertility (Hammond et al., 2016; E. Yang et al., 2022). Embryo cutting occurs when Cas9 and gRNA transcripts and proteins are maternally deposited into oocytes. Any wild-type alleles (at the drive or distant target site), especially paternal wild-type alleles after fertilization, can be cleaved, which always results in resistance allele formation at this stage (since the window for homology-directed repair is over). This process impairs the drive's spread much more than germline resistance alleles because female drive progeny will be sterile, and male drive progeny will be unable to perform drive conversion. Similarly, undesired Cas9 expression in somatic cells, regardless of whether it results in drive conversion or resistance allele formation, will disrupt the wild-type alleles needed for female fertility, at least in some cells. Depending on where and when the fertility target gene is needed, even necessary germline expression could induce a similar fitness cost in female drive heterozygotes (or any female with at least one drive allele and at least one wild-type fertility target gene).

To assess the effects of the embryo cut rate and somatic expression fitness cost under moderate drive conversion, we model a total cut rate of 0.9 and drive conversion rate of 0.5. These parameter choices mimic values that have typically been achieved in species that seem recalcitrant to gene drives such as *D. melanogaster* and *Aedes* mosquitoes (J. Champer et al., 2017; M. Li et al., 2020; Reid et al., 2022b; E. Yang et al., 2022). Varying the embryo cut rate and somatic expression parameters over their full range (Figure 5.5A), we see that the haplolethal two-target drive is sensitive to high embryo cut rates, whereas the haplosufficient two-target drive is not strongly affected by this. Both are affected negatively by female

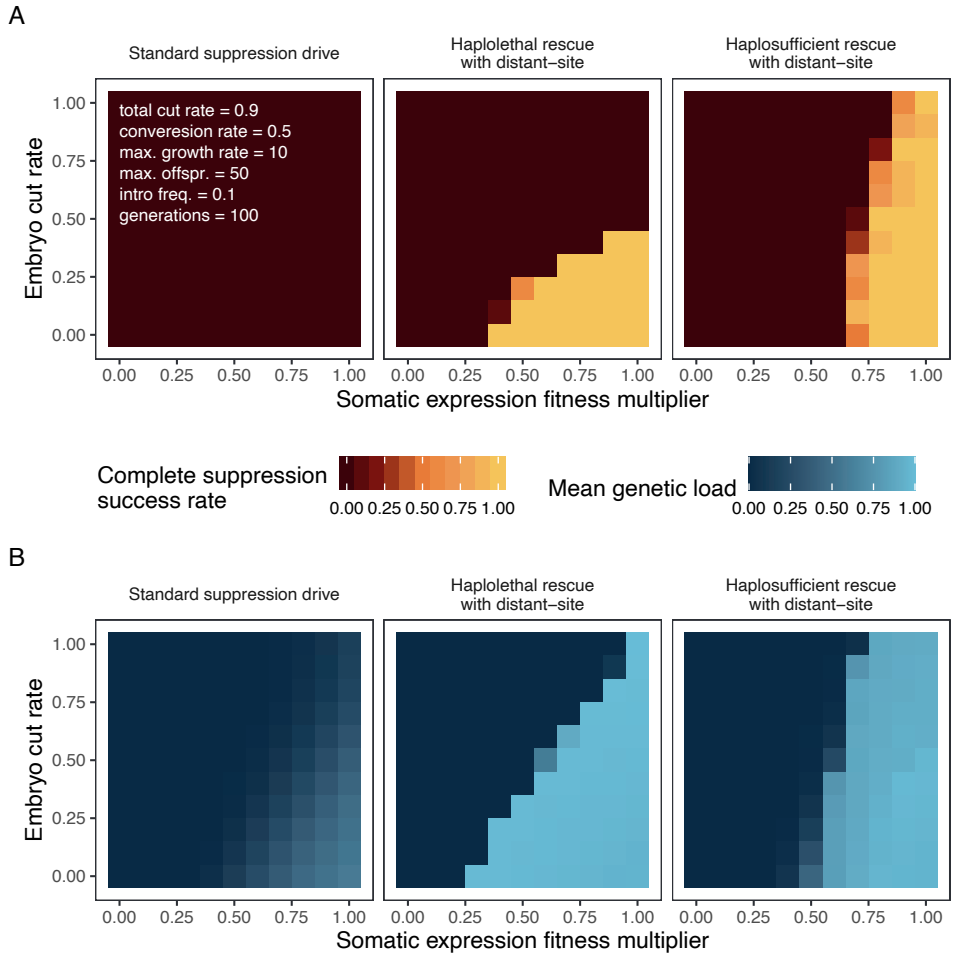


Figure 5.5. Drive performance under varying somatic and embryonic drive activity. A) Complete suppression success rate and B) mean genetic load under variable female fitness in drive heterozygotes from somatic expression fertility effects and variable embryo cut rates in the progeny of drive females. The complete suppression success rate is calculated as the fraction of simulations in which population elimination occurs within 100 generations after drive introduction. For A, the introduction frequency of gene drive heterozygotes is 0.1, and the total population capacity is 100,000. The total cut rate is fixed to 0.9, and the drive conversion rate is 0.5. For each combination of parameters, we ran 10 model repetitions.

heterozygote fitness costs, which prevent success more easily for the weaker haplosufficient drive. Looking at genetic load (Figure 5.5B), the standard suppression drive starts out weak, with increases in the embryo cut rate having roughly half the effect of reduction of female fitness. The two-target drives have a larger area of high genetic load compared to the area of complete suppression success, representing areas where the drive is still able to increase and eventually reach higher genetic load, but not within the time frame of the normal simulations. Higher initial release frequencies could still allow these drives to succeed in a shorter time frame.

To further show drive dynamics besides the eventual genetic load, Figure S5.5 shows population size over time after the gene drive introduction. The standard suppression drive cannot perform well with these parameters, so it always reaches an equilibrium population size, which is higher with increasing embryo cutting and fitness cost. With no somatic fertility cost and no embryo cutting, both two-target drives suppress the population rapidly. With an increasing embryo cut rate, all drives are slower for all three drives, but the haplolethal two-target drive is especially sensitive to this, losing all suppressive power (and the ability to increase in frequency at all) when the embryo cut rate is high. This is because high embryo cutting at the drive's site results in immediate removal of drive alleles in the progeny of females (all progeny with non-functional resistance alleles at this site are nonviable).

With decreasing drive female fitness from somatic Cas9 cleavage, all drives are slower to suppress the population. Eventually, the drives lose the ability to increase in frequency in the first place, with the exact point being dependent on the drive (Figure S5.6). With low embryo cutting, the haplolethal drive remains successful for lower female fitness values, and this trend is reversed for higher embryo cutting.

5.3.3 Functional resistance alleles

Functional r1 resistance alleles have been shown to be a major challenge for many gene drives, so we investigate how well these can be handled by the two-target drive design. We only model r1 alleles at the homing site of each drive, and not at the distant site. The rescue modification drives can suffer from r1 alleles that form due to incomplete homing, where only the rescue sequence is copied, but not the rest of the gene drive (S. E. Champer et al., 2020). Thus, even with multiplexed gRNAs, there may be a lower limit for functional

resistance for these sorts of drives. For a standard suppression drive, this is not an issue. However, while multiplexing can likely reduce functional resistance to low levels (E. Yang et al., 2022), this could reduce the drive conversion rate with more than 2-4 gRNAs, or with gRNAs that are further apart, both of which could cause drive failure due to lack of genetic load, thus leading to practical limits for multiplexing that may still permit functional resistance. At the distant site, multiplexing is more flexible because either homology-directed repair or end-joining can achieve the desired result. This could allow for more gRNAs that target the most highly conserved sites throughout the target gene, largely eliminating the chance to form functional resistance alleles, much like CRISPR toxin-antidote drives (J. Champer, Kim, et al., 2020).

We compare the drives in a range of parameter space where all are always successful in the absence of r1 alleles (Figure S5.8). We see that at relatively low r1 rates, the standard suppression drive is the first to succumb to functional resistance allele formation in the majority of repetitions (Figure 5.6). This is because r1 alleles provide immediate benefit, directly allowing females to be fertile, which results in rapid increase in frequency of the r1 allele at low population density (Figure S5.8 and Figure S5.9). Thus, if a resistance allele forms, it only needs to avoid stochastic loss for a small number of generations before it will prevent population elimination. Only the haplolethal two-target drive remains successful in most repetitions with a high r1 rate, where both other drives fail in most or all repetitions. The haplosufficient two-target drive remains successful in most replicates at intermediate r1 rates, whereas the standard suppression drive fails at relatively low r1 rates. In both two-target drives, r1 alleles have only a modest advantage over the drive allele, and this is indirect. Individuals will not experience the modest female heterozygote fitness cost if they have only r1 and wild-type alleles at the drive site, and the r1 allele will not itself disrupt the female fertility gene for progeny, potentially increasing its chance of being passed on to a fertile female. However, the drive will reach high frequency (Figure S5.9), meaning that most r1 alleles will be together with drive alleles, limiting their advantage and thus doing little to prevent suppression, which only requires one drive allele to disrupt the distant-site female fertility gene.

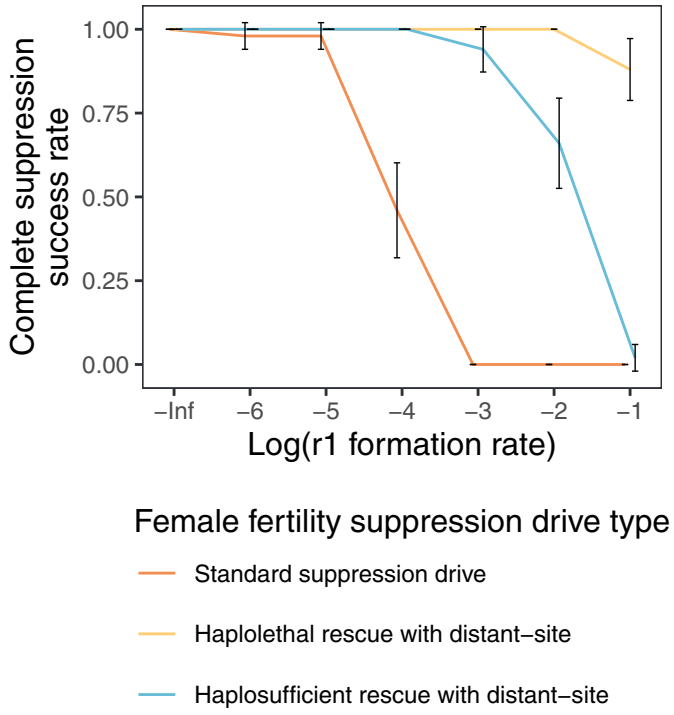


Figure 5.6. Impact of functional resistance alleles on drive performance. Successful population elimination under various functional resistance allele formation rates. The r1 formation rate is the relative rate of resistance alleles that are functional, rather than non-functional r2 alleles. The model is run for 100 generations after an introduction of drive heterozygotes at 0.01 frequency into a population of 100,000. To compare the drives where they were all successful in the absence of functional resistance alleles, we used a total cut rate 1.0, drive conversion rate 0.9, somatic expression female fertility fitness effect of 0.9, and an embryo cut rate of 0.1 in the progeny of female drive carriers. For each combination of parameters, we ran 50 model repetitions. Error bars show the 95% confidence intervals around the mean.

5.3.4 gRNA saturation

Because the two-target drives need double the amount of gRNAs, the drive as a whole may suffer from additional gRNA saturation compared to a standard homing suppression

drive. This means that the cutting efficiency of each individual gRNA is reduced due to limited amount of Cas9 protein. Gene drives using multiplexing to avoid r1 alleles already face this challenge (S. E. Champer et al., 2020), which would be amplified in two-target drives. We do not model multiplexing explicitly, but instead assume that an equal number of gRNAs will be used for the homing site and the distant site, thus allowing us to reduce the cutting efficiency at each site proportionally. Here, we calculated the genetic load for each drive with various cut rates and gRNA saturation factors (Figure S5.12). The gRNA saturation factor is the relative Cas9 activity level with unlimited gRNAs (spread equally between all the gRNAs), with "1" being the activity in the presence of a single gRNA (S. E. Champer et al., 2020). A saturation factor of 1 thus means that the total cut rate is immediately split between any number of gRNAs, while a rate of infinity (our default in previous simulations) means that the cut rate at each gRNA target is the same as the cut rate of a 1-gRNA system.

The standard suppression drive is not impacted by this model of gRNA saturation because this drive only targets a single site (it can be thought of as the "baseline" in this scenario, even if it would have multiple gRNAs in practice) (Figure S5.12). The two-target drives are both impacted by gRNA saturation, which reduces the genetic load a moderate amount if the gRNA saturation factor is low. This reduction is low if the total cut rate is high (Figure S5.13). For the haplosufficient drive with a high cut rate, gRNA saturation actually has a positive effect on the drive because its optimal cut rate is somewhat below 1 (Figure 5.4 and Figure S5.14). The current best estimate of the gRNA saturation factor is 1.5 (S. E. Champer et al., 2020), though this could potentially vary substantially between systems. Overall, gRNA saturation is potentially problematic, though the two-target drives would still usually be expected to have higher power than a standard suppression drive, especially when the total cut rate is high.

5.3.5 Test of two-part gene drive in *Drosophila melanogaster*

We construct the two-target gene drive design in *D. melanogaster* by reusing two previously built gene drives, a standard female fertility suppression drive and a haplolethal modification drive, and combining them (J. Champer, Yang, et al., 2020; E. Yang et al., 2022). The homing construct is integrated in the haplolethal gene *RpL35A*, whereas the distant target site is the haplosufficient female fertility gene *yellow-g* (Figure 5.7A). This drive was

constructed based on another successful drive reported previously (J. Champer, Yang, et al., 2020), by adding extra gRNAs targeting *yellow-g* from a previous suppression drive (E. Yang et al., 2022) to convert the original modification drive into a suppression drive.

Two independent EGFP-marked drive lines (named as line 1A and line 1D) were generated. These drive lines were respectively crossed to a DsRed-marked *nanos*-Cas9 line to produce heterozygous individuals carrying one copy of the drive and one copy of the Cas9 allele, after which these heterozygotes were crossed to w^{1118} flies for assessing drive efficiency. The drive inheritance rates of Line 1A were 78% for drive males and 71% for drive females, while the inheritance rates of Line 1D were 76% for drive males and 67% for drive females. In comparison, the inheritance for the drive in the absence of Cas9 were all approximately 50%. Our fitted model indicates significant effects of cross (control or drive) ($\chi^2(1) = 112.60, p < .001$) and the sex of the drive parent ($\chi^2(1) = 13.89, p < .001$), but not between lines or any interactions (Figure 5.7B and Source Data Set S1). The drive inheritance rates were much lower than the original haplolethal homing drive, where drive inheritance rates for both male and female heterozygotes were 91%. This difference is likely caused by gRNA saturation, meaning that the two gRNAs of the homing drive had lower cut rates because they shared the same amount of Cas9 with the additional four gRNAs targeting *yellow-g*. Besides the difference between males and females, the two gRNA lines performed slightly differently in drive conversion rate as well.

5.3.6 Cage experiments

To characterize the population suppression activity of this drive, cage experiments were set up by mixing drive carriers (heterozygous for the drive allele and homozygous for Cas9) and a small fraction of Cas9 homozygous flies. These cages were followed for several discrete generations, measuring drive carrier frequency by phenotyping all adults. Though there was some stochasticity in population size due to the single bottle nature of the cages, the dynamic of the drive was clear. Drive carrier proportions of most cages quickly decreased within five generations (Figure 5.8A). The population size for the two lower frequency releases was unaffected, and for one of the high release frequency cages, the population size recovered after an initial reduction (Figure 5.8B, Source Data Set S2). However, the other high frequency release cage showed complete population suppression in the second generation even though drive carrier frequency was declining. In the suppressed population, the

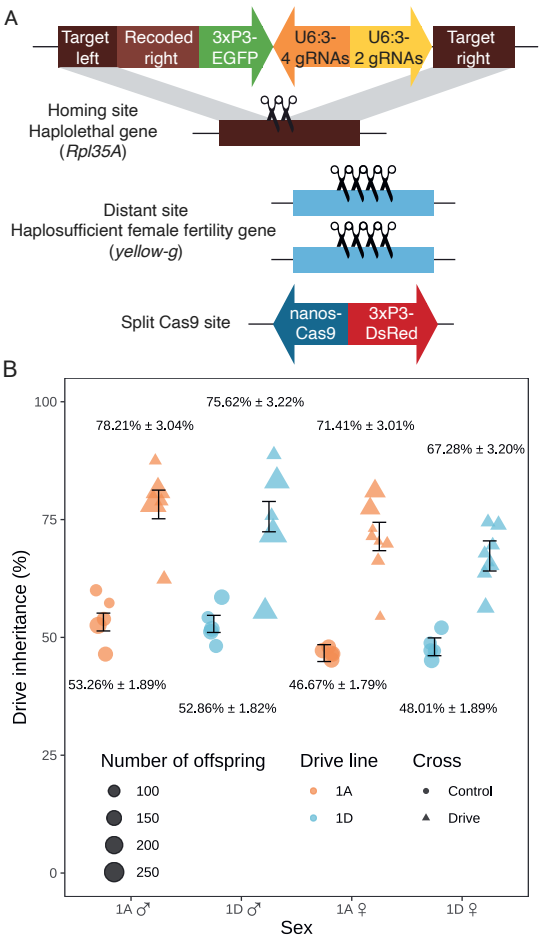


Figure 5.7. Drive construct and inheritance rates. A) The drive allele is inserted into the haplolethal gene *RpL35A* and contains a recoded version as rescue to avoid gRNA cleavage. It is expected that only offspring inheriting two functional copies of *RpL35A* (which can be either a drive or wild-type allele) can survive. The two scissors at the *RpL35A* site show the gRNA sites for cleaving and homing, while the four scissors at the *yellow-g* site show the gRNA sites for disrupting *yellow-g*, a haplosufficient female fertility gene located at a distant site. B) The drive inheritance rate indicates the percentage of offspring with EGFP fluorescence from the cross between heterozygotes (containing one copy of drive and one copy of Cas9) and w^{1118} flies (without drive and Cas9). The size of each dot represents the total number of offspring in that batch, and whiskers indicate the standard error of the mean. For each group from left to right, n = 691, 760, 751, 968, 780, 777, 702, and 800 flies.

five remaining flies all happened to be females, whereas this was not the case in the population that recovered. There, the five flies in that generation consisted of four females (one of which lacked the drive) and one male (also without the drive), allowing the population to grow immensely in the next generation. This result implies that the distant-site suppression drive was functional, though overall efficiency was low due to drive fitness costs outweighing biased drive inheritance. It also confirms that near-fixation of a gene drive will not be enough to suppress a population of *D. melanogaster*, given their ability to produce many offspring from just a few remaining individuals under the conditions of these cages.

5.3.7 Resistance alleles and fertility

While functional resistance is a possible explanation for the rapid decline of a suppression drive from high frequency, we considered this unlikely due to the use of multiplexed gRNAs and previous studies in larger cages with similar drives finding no evidence of functional resistance (J. Champer, Yang, et al., 2020; E. Yang et al., 2022). To explore the possible reason for poor cage performance, a fertility test was conducted to assess the fitness of our drive carriers (which was found to be reduced in both drives this two-target drive is based on, but not to the extent of eliminating them from cages, J. Champer, Yang, et al., 2020; E. Yang et al., 2022). We observed a considerable reduction in egg viability in females compared to males, particularly in those carrying the drive. For females with the drive, 24% of eggs were viable in line 1A, and 19% were viable in line 1D. In contrast, for males carrying the drive, 96% of eggs were viable in line 1A, and 84% were viable in line 1D. In individuals not carrying the drive, the average egg viability is similar between males and females (Figure 5.9 and Source Data Set S3). Interestingly, outliers in both drive carrying and non-drive carrying females indicate that a similar mechanism might be behind the reduction in egg viability. Our fitted model indicates significant effects of the sex of the drive parent ($\chi^2(1) = 16.62, p < .001$), and line (1A or 1D) ($\chi^2(1) = 8.12, p = .004$), as well as of interactions between cross (drive carrying or not) and sex of drive parent ($\chi^2(1) = 12.42, p < .001$), and between cross and line ($\chi^2(1) = 5.12, p = .024$). We collected 16 and 18 drive daughters from drive females (which were offspring of the above cross) of line 1A and line 1D, respectively, and crossed them to w^{1118} males. 87.50% from line 1A and 77.78% from line 1D were sterile (Table S5.1), which was likely caused by resistance allele formation in the embryo at the *yellow-g* target site due to maternal deposition of Cas9 and gRNAs. Indeed, sterile females were randomly collected for sequencing, which indi-

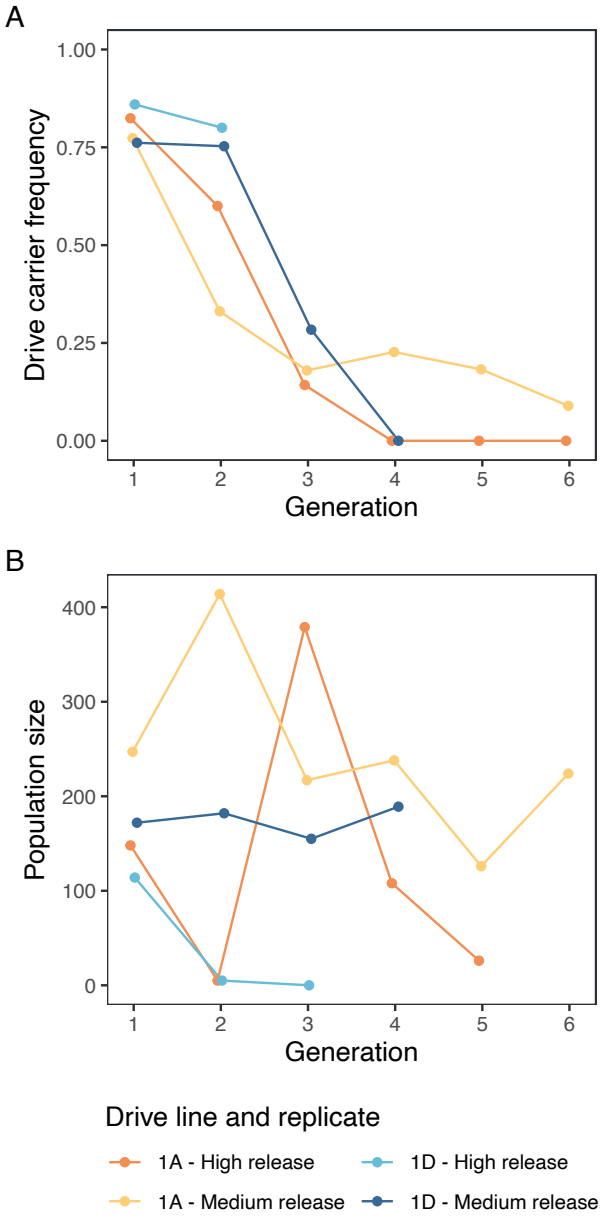


Figure 5.8. Small cage experiments. Heterozygous flies carrying one copy of the drive allele and two copies of Cas9 were used to generate progeny for "generation zero" of four small cages (the two medium release cages also included a non-drive female that was also homozygous for Cas9). A) Drive carrier frequency and B) population size in each generation.

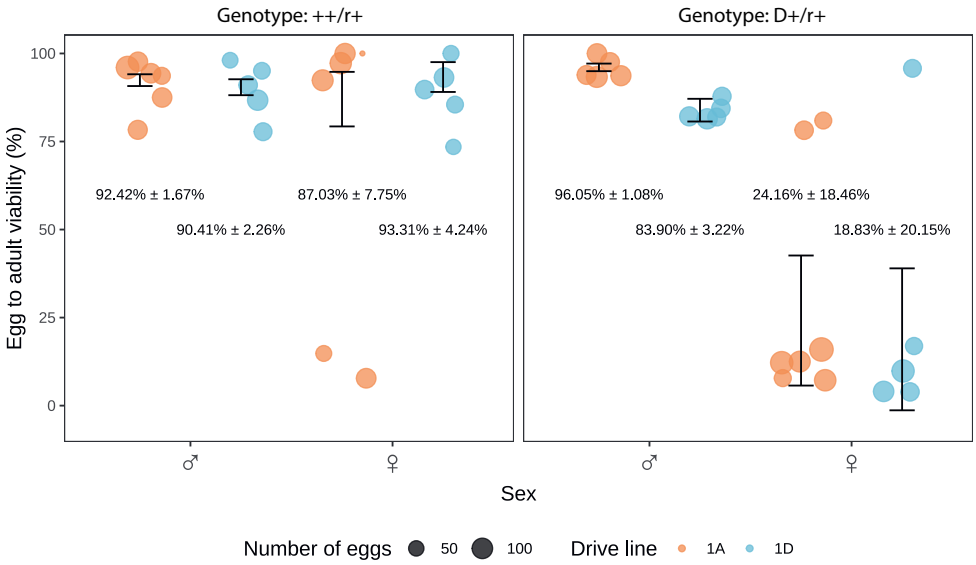


Figure 5.9. Egg viability test. The individuals tested in this experiment were all homozygous for Cas9, but different in the drive site and distant site, for which genotypes are listed at the top. "+", "D" and "r" respectively represent wild-type allele, drive allele and non-functional (*yellow-g* target site) resistant allele. Note that while cut rates at the *yellow-g* site were high, it is possible that a small fraction of flies marked as having an "r" at the target site were in fact homozygous for wild-type at this site. The size of each dot represents the total number of eggs laid in that batch, and whiskers indicate the standard error of the mean. For each group from left to right, n = 534, 361, 463, 332, 453, 404, 681, and 434 eggs.

cated 100% cutting in the *yellow-g* target site. Another 16 non-drive progeny from a drive female and Cas9 male cross were randomly collected for sequencing, showing 87.5% (14 out of 16) cutting in *yellow-g*. These results indicate that the poor cage performance in this study is likely due to fitness cost of female drive heterozygotes together with high levels of embryo cutting and subsequent resistance allele formation at both the female fertility site and the haplolethal drive site, though it is currently unclear to what extent each of these factors contributes.

In addition to the egg to adult viability, we measured the drive inheritance of these off-

spring. The drive inheritance rate of line 1A was 67.82% ± 2.83% in drive males and 74.93% ± 3.73% in drive females, while it was 75.99% ± 2.67% in drive males and 79.47% ± 4.62% in drive females in line 1D (Figure S5.16). The drive inheritance rates of these flies were somewhat higher than our previous test where drive heterozygotes had only a single copy of Cas9, except for drive males of line 1A. The reason for this anomaly is unclear, particularly considering that lack of Cas9 appears to be a limiting factor in the performance of the drive compared to the original modification drive it is based off. Additionally, although drive inheritance rates in the previous test were higher in drive males than drive females, this difference was not seen in this later drive conversion test.

5.3.8 Whole-genome sequencing

To further investigate the difference in performance between lines 1A and 1D, as well as the generally lower than expected homing efficiency, we sequenced the genome of both drive lines using Nanopore sequencing, because initial screening indicated the presence of repeated regions. For line 1D, a single contig covered the entire gene drive, showing that although the line contained the full construct as expected, the insertion was duplicated, including the cloning vector in between the two copies (Figure S5.17C). This means that line 1D contained an extra copy of the distant-site gRNAs cassette. For line 1A, there were five separate contigs covering the gene drive. We found that the distant-site drive construct was incorporated thrice, with two vector backbones in between, and with inversions between two gRNA cassettes (Figure S5.17B). This was likely caused by recombination between gRNA cassettes during transformation. Line 1A contained four gRNA cassettes, but both distant-site ones were only partially present (two out of four distant-site gRNAs were eliminated due to the recombination).

5.4 Discussion

In this study, we analyzed the possibility of converting a modification to a suppression drive by addition of gRNAs targeting a female fertility or other essential gene without rescue. We found via modelling that this can substantially increase the genetic load (suppressive power) of the drive compared to a similar standard suppression drive, unless the drive conversion rate is already very high. Instead of the genetic load being mainly determined by the drive conversion rate, it is determined by the total germline cut rate in these two-target

drives. We then tested such a drive in the model organism *D. melanogaster*, which successfully biased its inheritance while cutting the distant female fertility gene target at high rates. However, fitness costs in drive heterozygous females were quite high for this drive, which prevented success in most cage replicates despite high release frequency. Nevertheless, two-target drive systems may be quite desirable tools for suppression of pests where high drive conversion is difficult to achieve but not high cut rates. Such high drive conversion has only been consistently achieved in *Anopheles* mosquitoes, while several other species have had substantially lower or inconsistent drive conversion, such as *Drosophila*, *Aedes*, and mice (M. A. E. Anderson et al., 2023; J. Champer et al., 2017; Kandul et al., 2020; M. Li et al., 2020; Oberhofer et al., 2018; E. Yang et al., 2022). In these, relatively high cut rates have often been achieved when drive conversion is low, which would be necessary for the two-target drive design to work in these species.

In the two-target suppression drive, population suppression is largely decoupled from drive frequency increase. This is in contrast to the standard homing suppression rive, where the drive allele itself is mainly responsible for disrupting the suppression target. Thus, the two-target drive can keep increasing in frequency and eventually cut most female fertility target alleles. The standard suppression drive reaches an equilibrium that allows many wild-type alleles to remain, mostly accounting for its genetic load. Though the two-target suppression drive allows for higher suppressive power, it still needs to be able to increase in frequency in the first place. Thus, drive conversion must be sufficiently high to overcome fitness costs due to somatic expression and embryo cutting (the latter of which is mostly important at the distant site, though it could have a substantial negative effect at the drive site if the drive targets a haplolethal gene there). These performance parameters are still highly dependent on the Cas9 promoter and to a lesser extent, the target gene. For the target gene, additional possibilities are available for two-target drives because they can keep high genetic load even when targeting X-linked female fertility genes.

While our modeling results are promising for two-target drives that lack high drive conversion rates, it should be noted that drive conversion is still essential for determining the speed at which the drive's frequency will increase, which determines the time to population elimination. Though this system can increase the genetic load, such drives will still take longer to eliminate a population than a drive with high drive conversion efficiency (regardless of whether such a drive is a standard suppression drive or a two-target system). In more com-

plex environments such as two-dimensional continuous space, this slowed rate of increase could potentially result in failed suppression due to the "chasing" effect, where wild type individuals migrate back into an area previously cleared by the gene drive, resulting in cycles of suppression and re-establishment (S. E. Champer et al., 2021). However, this same study included a powerful but slow drive that avoided this effect, which may be analogous to a two-target system. Additional modeling is thus needed to explore the outcomes of two-target drives in complex scenarios (Zhu & Champer, 2023).

It should be noted that like standard homing drives, measures to limit or localize this drive may be necessary. The designs modelled in this study could be threshold-dependent under certain narrow parameter combinations (for example, certain somatic expression fertility effects and embryo cut rates). Outside of those ranges, such as standard homing drives, they could be designed to be self-limiting or threshold-dependent by incorporating a daisy-drive (Noble et al., 2019) or tethered design (Metzloff et al., 2022). Additionally, the fact that two-target drives target multiple loci could have interesting potential for targeting a specific population only based on locally fixed alleles (Sudweeks et al., 2019). In fact, the locally fixed allele strategy would be easier to implement with a two-target drive because the gRNAs targeting the female fertility gene would not need to be close together to achieve high drive conversion efficiency.

In our experimental demonstration, the constructs functioned in terms of drive conversion while cutting the distant site at high rates. However, drive efficiency at the homing site was considerably lower than the original modification rescue drive (J. Champer, Yang, et al., 2020), for which duplications in our drive lines could be the cause. Although the drive seems stable in our lines based on the pooled genomic data of 25 individuals, it may not have been during drive conversion due to duplication of homology arms, resulting in loss of some regions. More importantly, during homology directed repair when the gene drive induces a double stranded break, DNA from the homologous chromosome might form secondary structures due to the repeats in the gene drive. This could potentially obstruct the repair machinery and cause replication stress (Saada et al., 2018), reducing the relative efficiency of homology-directed repair. In another recent homing rescue drive, a low homing efficiency was observed, which increased when a repeated region was replaced with a similar element that contained several mutations (Hou et al., 2024). Considering these, it seems advisable to avoid repeated regions as much as possible in gene drive constructs,

which could be done readily by simply combining the gRNAs into a single cassette or using different gRNA promoters.

Besides the structural problems, the lower efficiency could also be due to Cas9 saturation from the additional gRNAs targeting the distant site, since we used the same Cas9 as the original homing rescue drive (J. Champer, Yang, et al., 2020). The fact that line 1A contained more homing gRNAs (both proportionally and absolutely) could partially explain the higher inheritance rate we observed. Since cut rates were very high at the distant-site, gRNA saturation likely had a smaller impact there. The cut rate at the homing site would be expected to decrease more than at the distant site, especially because the cut rate for the original modification drive was not high in the first place (J. Champer, Yang, et al., 2020), at least compared to other published drives in *D. melanogaster*. These issues could be more readily avoided using entirely new designs with balanced numbers of high-activity gRNAs for both target sites. In general, however, the fact that two-target drives use a higher number of gRNAs than standard homing drives could be a potential weakness due to gRNA saturation reducing the cut rate at each site. We expect this reduction to be generally limited though, because cutting rates are high for many organisms, thus reducing the impact of gRNA saturation.

Arguably more problematic than the homing efficiency, the high fitness cost in female heterozygotes prevented success in population cages, and embryo cutting at both the drive site and the distant-target site further contributed to drive failure. This fitness cost was expected to an extent because our two-target drive is based on a suppression drive showing reduced fitness as well (E. Yang et al., 2022). In the haplolethal modification drive, drive carrier males showed no significant reduction in offspring egg-to-pupa survival, but females did show a significant effect (J. Champer, Yang, et al., 2020). This fitness reduction would represent an addition fitness cost in our two target drive and is likely from non-functional resistance alleles rendering offspring non-viable. If formed in the germline, these alleles would not have significant negative effects, but if formed in the embryo, it could further contribute to reduced drive success. In the earlier standard suppression drive targeting *yellow-g*, at first, offspring from neither male or female drive parents showed a significant reduction in the egg-to-pupa survival rate, but later crosses with drier food conditions did show a significant reduction in egg-to-pupa survival rate, and inference from cage experiments showed even higher fitness costs. This fitness cost likely indicates either leaky somatic

expression or disruption of *yellow-g* too early in the germline where it still might need to function. Together, these factors could all contribute to the more severe fitness cost for female drive carriers with our two-target drive, though we currently do not know what factor contributes to what extent. In the original suppression drive, offspring of drive carrier females were found to be sterile at high rates. This infertility indicates significant embryo activity, which also had a similar negative effect on the two-target drive. In the two-target drive, gRNA saturation could have slightly ameliorated these effects due to the presence of more gRNAs and cut rates being slightly reduced, but the different genomic site may change this, and reduction in drive conversion efficiency for similar reasons would likely outweigh any benefits.

All of these fitness costs will likely be similar for two-target drives targeting different genes using *nanos*-Cas9, with the exception of *yellow-g* potentially being required in the early germline. Therefore, finding promoters that are exclusively expressed in the (late) germline is essential for future development of two-target drives (and any suppression drive for that matter), and promoters with little to no embryo activity are also preferred. In the standard suppression drive, however, the higher drive conversion rate (and lack of embryo cutting at the haplolethal target) still allowed it to reach a moderate equilibrium instead of declining to zero. If the two-target drive in our study had similar or even somewhat lower drive conversion, but lower fertility cost or embryo resistance allele formation, our model indicates that it would have been able to increase in frequency in the population and eventually eliminate it with high genetic load. Even in current form, our drive could potentially be successful with repeated releases, analogous to a more powerful form of sterile insect technique, which is far less powerful than a gene drive but still potentially useful in several situations due to its self-limiting nature (Spinner et al., 2022).

Overall, we have shown that for scenarios of low to moderate drive conversion and high total germline cut rates, two-target drives may offer substantially increased suppressive power. With further efforts to find better Cas9 promoters and target gene combinations with lower fitness costs, this unique drive design may unlock the potential for strong population suppression in many scenarios.

5.5 Methods

5.5.1 Ethical Statement

This research complies with all relevant ethical regulations and was approved by the Peking University biosafety office.

5.5.2 Modelling

We use a stochastic, individual-based model with non-overlapping generations in a randomly mating population of fixed carrying capacity. We use the population genetics modelling software SLiM version 4.0.1 (Haller & Messer, 2022) in combination with R version 4.1.0 (R Core Team, 2025). Our code can be found on GitHub at https://github.com/NickyFaber/Two-target_drive.

Drive types

We have modelled 10 different types of homing suppression drives. For ease of visualisation, we have chosen to show the three most representative drives in the main results. A list of the rest of the drives and their modelling results are in the Supplementary Materials.

1. Drive with female fertility target
2. Haplolethal rescue drive with distant-site female fertility target
3. Haplosufficient rescue drive with distant-site female fertility target

For a detailed explanation of the mechanics and phenotypes of these three drives, see the results. To model each drive, we include two loci in SLiM: the homing site and an optional distant-site. At the homing site, there are four potential alleles: wild type, drive, r1 (functional resistance), and r2 (non-functional resistance). At the distant site, we only model wild-type and r2 alleles (full freedom to choose any set of gRNAs is assumed to reduce functional r1 resistance to negligible levels, see results). The structure and steps of the model are described below.

Model structure

We have used previous work by Champer et al. (2020) as a starting point for our modelling (S. E. Champer et al., 2020). That study modelled complex drive activity in the germline, including gRNA multiplexing (the number of gRNAs used to target a site), timing of drive activity (early or late germline), and gRNA saturation (the effect that a limited supply of Cas9 reduces the cutting efficiency at each targeted site with an increasing number of targets). Since our objective is to compare various gene drive designs focusing on genetic load instead of on resistance alleles, we have removed some of those complexities for this study. We model the population and introduction of the gene drives as follows:

- Generation 1: Population initialization
- Generation 1-10: Population equilibration without gene drive
- Generation 11: Introduction of heterozygous gene drive individuals
- Generation 111 or 161: End of the model. As default, we run the model for 111 generations (100 generations with gene drive). When we calculate the genetic load, we run it for an additional 50 generations to increase the number of generations in which we can determine the equilibrium genetic load.

In each generation, the model executes the following steps in order (Figure 5.10):

1) Reproduction. In our model, the number of offspring that is generated by each female is a product of the fertility status of the female, her ability to find a mate, the carrying capacity, and the fitness of the female.

- Fertility status check. A female could be infertile due to the drive mechanism, both at the homing site and the distant site. These loci are both checked for the presence of non-functional alleles, that is, either a drive allele or an r2 allele. If the female has two of these in any combination at at least one locus, she does not generate any offspring.
- Selecting a mate. A female randomly selects a male from the population. If there are no males available, she does not generate any offspring.
- Generating offspring. In this study, we model density-dependent fertility (rather than density dependent mortality or a combination of the two). This is computa-

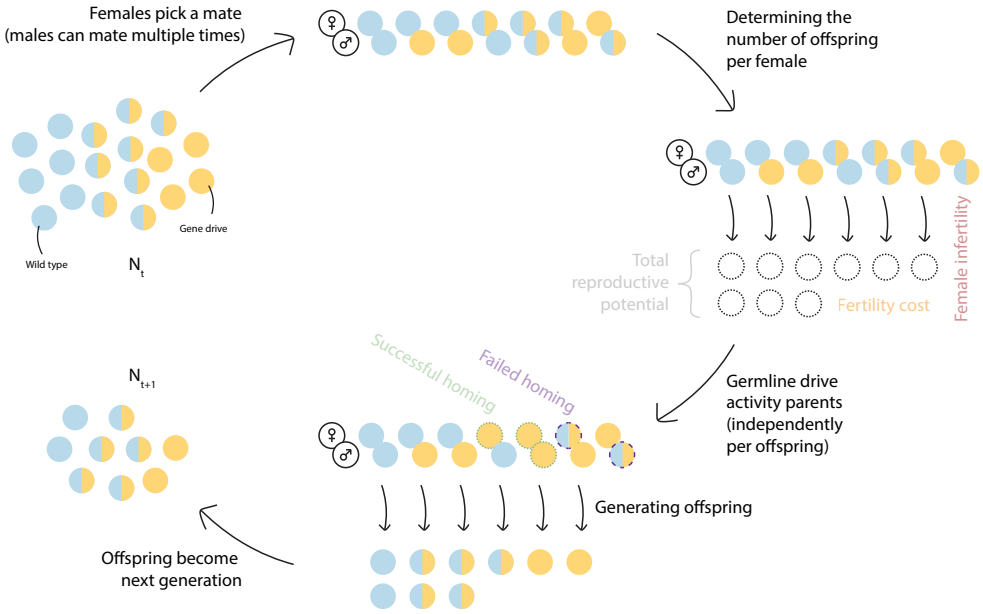


Figure 5.10. Model overview, showing a simplified sequence of steps the model goes through every generation. Each circle represents an individual, and its color shows the genotype. We show a simple homing gene drive with only wild type (blue) and drive (yellow) alleles. The two-target drives will show different dynamics dependent on their design (Figure 5.2). After each female picks a male for mating, we calculate the reproductive potential of these females. Then, based on drive genotype, females can be either completely fertile, or completely sterile (drive homozygotes), or partially fertile (drive heterozygotes). Offspring is generated, and for each individual offspring, Mendelian inheritance is modified by drive activity in the parental germline. This means that drive conversion could be successful for one offspring of a single drive parent, but not another. Additional drive activity from maternally deposited Cas9 is also modeled, but not shown in this figure. Finally, these offspring become the next generation of adults.

tionally efficient and still allows easy density regulation. See Dhole et al. (2020) for a discussion on how genetic load could translate to population size reductions for different species-specific scenarios (Dhole et al., 2020). Different models would be

more suitable for particular species, but genetic load itself would be dependent on the drive mechanism, regardless of the exact form of density dependence. For each female, the number of offspring (O) she generates in that generation (i) is based on a binomial distribution:

$$O_i = B(O_{max}, p_i), \tag{5.1}$$

where i is the current generation, O_{max} is the maximum amount of offspring per female, and p is the average fraction of this maximum amount of offspring that will be generated. This fraction is normally defined as $\frac{2}{O_{max}}$ (each female must generate two offspring to maintain the population at carrying capacity). However, our model includes two further influences. First, p can be increased when the population is below carrying capacity because offspring will have more resources to survive, or vice versa, which we call the carrying capacity factor (F_{CC}). Second, p can be decreased in female drive carriers due to somatic expression of the drive reducing fertility (because some wild-type female fertility alleles are disrupted in somatic cells where they are needed for fertility), which we call the somatic expression fertility factor (F_{SEF}). Thus, p is defined as:

$$p_i = F_{CC_i} * F_{SEF_i} * \frac{2}{O_{max}}. \tag{5.2}$$

The carrying capacity factor is defined so that at very low population densities, it is close to the maximum growth rate (r), at carrying capacity (K), it is close to 1, and above carrying capacity, it is between 0 and 1. This leads to a logistic growth curve:

$$F_{CC_i} = \frac{r}{(r-1) * \frac{N_i}{K} + 1}, \tag{5.3}$$

where N is the number of adults in the population.

Fertility scaling is done for females with at least one drive allele. Somatic expression of the drive can impact female fertility by prematurely disrupting wild-type fertility gene alleles. One of our modelled drives targets a female fertility gene at the homing site without rescue and the other two at the distant site. We only model somatic

expression fitness costs for the drive sites without rescue. The total fertility cost is calculated per female as being additive as follows:

$$F_{SEF} = m^{H_{wt}} * m^{\frac{1}{D_{wt}}}, \tag{5.4}$$

where m is the somatic expression fertility cost multiplier, from 0 (complete sterility) to 1 (no fertility cost), H_{wt} is the number of wild type alleles at the homing female fertility site (which can only be 0 or 1, since the other allele must be the drive), and D_{wt} is the number of wild-type alleles at the distant female fertility target. At the distant locus, individuals with two wild-type alleles will be less impacted by the fertility cost, since it is less likely that both copies of the fertility gene are disrupted due to somatic expression.

2) Gene drive activity. After all the offspring is generated, each offspring's genotype is modified based on the drive activity in the parents' germline. Drive activity in the parental germlines is modelled for each offspring independently. Cutting, homing, and the creation of resistance alleles is stochastic.

- Germline gene drive activity. For both parents of each offspring, the presence of the drive in their genome is checked. If at least one copy of the drive is present, the gene drive is active in the germline. First, the cut rate is a parameter in our model, but it can be impacted by gRNA saturation as follows:

$$P_{c_s} = 1 - (1 - P_c)^{\frac{s}{s+l-1}}, \tag{5.5}$$

where P_{c_s} is the cut rate adjusted for gRNA saturation, l is the number of loci, either 1 or 2 depending on whether the drive targets a distant site in addition to the homing site (we assume equal gRNA multiplexing at both sites), P_c is the global cut rate, and s is the Cas9 saturation factor, which can range from 1 (which means 1 gRNA is enough to saturate all Cas9 proteins, so the cut rate for each gRNA rapidly declines as more gRNAs are added) to infinite (which means no amount of gRNAs is enough to saturate the Cas9 proteins, so the cut rate at each gRNA remains the same regardless of the number of gRNAs). If a randomly generated number be-

tween 0 and 1 is higher than this cut rate, cutting occurs. We do this separately for the distant site alleles as well, if present.

At the distant site, cutting always results in an r2 allele. We made this choice based on the following assumptions: 1) with a high cut rate, wildtype loci on both chromosomes are likely to be cut simultaneously, reducing the chances of successful homology-directed repair, 2) alleles repaired back to wildtype can be re-cut multiple times, with a lower likelihood of homology-directed repair occurring each time, and 3) if the cut rate is high, most gene drive individuals will inherit one r2 allele from their drive parent, making it more likely that homology-directed repair will produce a second r2 allele. Our experiments in *D. melanogaster* showed that most offspring had r2 alleles at the *yellow-g* locus, indicating that homology-directed repair back to a wildtype allele is rare in our system.

At the drive locus however, homing can occur if there was successful cutting based on the homing success rate (P_h). The homing success rate is defined at the beginning of the model based on the conversion rate (P_{conv}), which is a parameter:

$$P_h = \frac{P_{conv}}{P_c}, \tag{5.6}$$

where P_{conv} can only be as high as P_c. If homing is successful, again based on P_h, the allele is converted to a drive allele. If there was cutting, but no homing, the locus is converted into an r1 allele with a probability equal to the r1 formation rate. Otherwise, it will become an r2 allele.

- Embryo gene drive activity. In the early embryo, we model maternal deposition of drive Cas9 and gRNAs. Potential cuts that occur here always result in a resistance allele (which can be r1 or r2 as above). The cut rate (P_{e_s}) is calculated the same as in Formula 5.5, except the exponent is additionally multiplied by a maternal deposition factor (d) that accounts for either a drive heterozygous or homozygous mother:

$$P_{e_s} = 1 - (1 - P_e)^{\frac{s*d}{s+l-1}}, \tag{5.7}$$

where P_e is the embryo cut rate, which is a parameter in our model. The maternal deposition factor is based on experimental data showing that the cut rate is higher

in embryos with a drive/wild-type heterozygous mother than expected due to drive conversion, so d is 1.83 and 2 for drive/wild-type heterozygotes and drive homozygotes, respectively. It would remain 1 for fertile drive/resistance allele heterozygotes (S. E. Champer et al., 2020).

- Offspring viability. After drive activity in the parental germline, inheritance, and embryo activity, we check if the offspring's resulting genotype is still viable. All drives that target either a haplolethal gene (where any non-functional resistance allele makes individuals non-viable) or haplosufficient gene (where only non-functional resistance allele homozygotes are non-viable) could result in non-viable offspring. These offspring are removed from the population.

3) Mortality. We model discrete, non-overlapping generations, so we remove the entire parental generation after offspring have been generated.

4) Calculating genetic load. Because our model is stochastic, populations can be suppressed if the genetic load is close to, but lower than the deterministic requirement, which is below 1 and depends on the maximum growth rate of the population. Therefore, in order to calculate genetic load with precision for drives with high genetic loads, we run a module in the model in which we artificially raise the number of offspring a female produces by dividing it by a certain bonus factor (that ranges between 0 and 1) (S. E. Champer et al., 2020). These are later corrected for when we calculate the genetic load. Thus, the bonus factor has no biological meaning, and is purely intended to precisely measure an outcome under stochastic variation. With this approach, the population is not eliminated unless the genetic load is practically 1, allowing for precise measurements of high genetic load for several generations when the drive is at its equilibrium frequency. This bonus factor (F_B) is calculated as follows:

$$F_{B_i} = \frac{N_{FF_i}}{N_{F_i}}, \tag{5.8}$$

where N_F is the number of females, and N_{FF} is the number of fertile females.

Then, in the next generation, the number of offspring calculated in Formula 5.1 is increased as follows:

$$O_{F_{B_i}} = \frac{O_i}{F_{B_i}}, \tag{5.9}$$

rounded to a whole number. Additionally, in genetic load simulations, gene drive carriers are introduced at 0.5 frequency, and the model is run for 150 generations after introducing the gene drive. The mean genetic load is calculated as the mean genetic load over the last 10 generations. The low-density growth rate and the maximum number of offspring are set a factor 10 higher than default (so 100 and 500 instead of 10 and 50, respectively) in order to calculate the genetic load precisely despite stochasticity.

5) Tracking outcomes of interest. Each generation, we calculate population size, genetic load, and genotype frequencies at both the homing site and the distant site. We calculate the genetic load (G) based on the observed and expected population size in the next generation (N_{i+1} and $N_{exp_{i+1}}$, respectively):

$$G_i = 1 - \frac{N_{i+1} * F_{B_i}}{N_{exp_{i+1}}}, \tag{5.10}$$

where F_B is the above mentioned bonus factor we apply.

N_{exp} is based on the number of females (N_F) and the carrying capacity factor F_{CC} defined in Equation 5.3:

$$N_{exp_i} = 2 * N_{F_i} * F_{CC_i}, \tag{5.11}$$

where F_{CC} is the same carrying capacity factor defined in Equation 5.3, and again multiplying by 2 because each female must generate two offspring to maintain the population at carrying capacity.

5.5.3 Experimental work

Plasmid construction

The starting plasmids TTTgRNAt and TTTgRNAtRNAi were used for building gRNA helper plasmids for knock-in (J. Champer, Yang, et al., 2020). The gRNA cassette used

in the donor plasmid was obtained from HSDygU4 (E. Yang et al., 2022). A two-step assembly process was done to generate donor plasmids (SI Appendix, Methods). Q5 High-Fidelity DNA Polymerase used for PCR and enzymes for digestion were purchased from New England Biolabs. PCR and restriction digestion products were purified with Zymo Research Gel DNA Recovery Kit, and plasmids were assembled by using HiFi DNA Assembly Cloning Kit and subsequently transformed into DH5α competent cells from TIANGEN. ZymoPure Midiprep Kit (Zymo Research) was used to prepare donor constructs for embryo injection. Oligo synthesis and Sanger sequencing was done by BGI Genomics. All the primers, plasmids, and construction procedures used in this study can be found in can be found in the Supplementary Information. Plasmid maps are available on GitHub (https://github.com/NickyFaber/Two-target_drive) in GenBank format created using ApE software (Davis & Jorgensen, 2022).

Generation of transgenic lines

All of the transgenic lines were generated via CRISPR/Cas9 homology directed repair. *D. melanogaster* injections were completed by UniHuaii, with a mixture consisting of the donor plasmid, Cas9 helper plasmid, and gRNA helper plasmid targeting the insertion site. To be specific, the donor plasmid HSDrgU2U4v2 (518 ng/ul) was injected into AHDr352v2 flies (J. Champer, Yang, et al., 2020) along with TTTrgU2t (150 ng/ul), which provided gRNAs for transformation, and Cas9-expressing helper plasmid TTChsp70c9 (450 ng/ul) to generate the drive line. The other donor plasmid SNc9NR (506 ng/ul) was injected into w^{1118} flies along with helper plasmids BHDabg1 (100 ng/ul) and TTChsp70c9 (459 ng/ul) to construct a *nanos*-Cas9 line. Surviving G0 flies were crossed to w^{1118} flies, and G1 adults were screened for transgenic inserts based on the presence of green or red fluorescence in the eyes. Flies were reared in an incubator at 25°C following a 14/10-h day/night cycle.

For phenotyping, flies were first anesthetized with CO_2 and then screened for fluorescence using the NIGHTSEA system (SFA-GR). The homozygosity of flies was scored by the fluorescence intensity and confirmed by sequencing.

Drive conversion

Drive (gRNA-expressing line) males were crossed to Cas9 females to generate heterozygous offspring, which was subsequently out-crossed to w^{1118}. The drive inheritance and sex of off-

spring were recorded. To confirm whether the distant target site in *yellow-g* was disrupted, individuals containing both drive and Cas9 alleles were randomly collected for genomic DNA extraction and genotyping. A fragment covering *yellow-g* target sites was amplified with primers 52_YGLeft_S_F3 and 54_YGRight_S_R6 (see GitHub DNA files).

Small cage study

Drive heterozygous males with red fluorescence from the Cas9 allele were crossed to homozygous Cas9 females for several generations to produce a line that was heterozygous for the drive and homozygous for Cas9 (D/+; Cas9/Cas9). Two experimental groups were set up with different initial release frequencies. In the higher release frequency group, four drive females were crossed to four drive males, while in the medium release frequency group, four drive females were crossed with four drive males, and one Cas9 virgin female was crossed to Cas9 males. Thus, the high drive frequency release (generation number 0) was 0.5 (1.0 carrier frequency), and the medium drive frequency release 0.4 (0.8 carrier frequency). These adults were allowed to mate in vials for one day before moving females into a separate bottle for oviposition. Females were allowed to lay eggs (which represented "generation 0") for three days and were then removed from bottles. When most pupae eclosed to adults, they were moved to a new bottle for a one-day oviposition before being removed and phenotyped. Hereafter, only one-day oviposition was conducted in each generation. The adults of each generation were scored for eye fluorescence phenotype and sex.

Fecundity and fertility test

To minimize batch effects caused by food quality or population density, flies with different genotypes used for this test were generated from the same parental cross and reared in the same bottle. First, males that were heterozygous for the drive and homozygous for Cas9 were crossed to Cas9 homozygous females, generating offspring with different genotypes. Next, these offspring were individually crossed to Cas9 homozygous males or females and allowed to lay eggs for three days. Adults in the same vial were moved to a new vial each day, and the number of eggs was counted in each vial. Offspring were allowed to hatch, and the egg-to-adult survival rate and adult phenotypes of these offspring were scored. Female drive offspring were randomly collected and crossed to Cas9 males, after which the sterile females were genotyped for the *yellow-g* distant site.

Statistics & reproducibility

All samples are listed in the figure captions and Source Data Sets. No data were excluded from the analyses. No statistical method was used to predetermine sample size. The experiments were not randomized because the experimenter needed to set up and track specific crosses. The investigators were not blinded to allocation during experiments and outcome assessment because the main experimenter needed to set up and track specific crosses.

Phenotype data analysis

For the statistical analysis of our experimental data, we fit generalized linear mixed-effects models containing our fixed experimental factors (genotype of the cross, drive individuals' sex, and transformed line 1A or 1D), as well as a random effect to correct for batch effects (each vial was considered an independent batch). Briefly, we fit two models with a binomially distributed response variable using a logit link and maximum likelihood estimation: one for gene drive inheritance and one for egg-to-adult survival. First, we fit a full model with all fixed effects and interactions and compared what random effect structure fit the data best. In the best model for gene drive inheritance, the random batch effect depended on the genotype (control crosses showed normal Mendelian inheritance, whereas the drive crosses showed increased variance in inheritance rates, which is likely an effect of pre-meiotic germline drive activity). In the best model for egg-to-adult survival, the batch effect depended on the sex of the drive parents (which is likely an effect of maternal deposition of Cas9 and gRNAs, though this data shows an interesting binary-like distribution). Then, we sequentially removed the least significant fixed effect from each model (starting with interactions) until only significant effects were left. To calculate the mean and standard error for each group, we fit a model with the same random batch effect, but with each group as a fixed effect. The analysis was performed using R (4.4.0) with support from the glmmTMB (1.1.9) (Brooks et al., 2017) and DHARMa (0.4.6) (Hartig, 2017) packages. The code and model summaries are available on GitHub (https://github.com/NickyFaber/Two-target_drive).

Whole genome sequencing

From line 1A and 1D each, 25 flies were pooled for DNA isolation using QIAGENs DNeasy Blood & Tissue Kit. A Nanopore library was prepared and sequenced on a MinION Mk1C (Oxford Nanopore). We used built-in adaptive sampling software to enrich for the 8Mb region around the gene drive on chromosome 3R (Payne et al., 2021). An overall sequencing depth of 40-50X coverage was reached in non-enriched regions of the genome, while around 70X coverage was obtained in the enriched region. Base-calling was done using Oxford Nanopore's basecaller Dorado (Oxford Nanopore Technologies, 2023), *de novo* genomes were assembled using Flye software (Kolmogorov et al., 2019), and reads were mapped to validate and manually curate the assembly around the gene drive sequences using IGV (Thorvaldsdóttir et al., 2013). Annotation of the consensus sequence was done manually in ApE (Davis & Jorgensen, 2022). Annotated sequences for both lines are available in GenBank format on GitHub (https://github.com/NickyFaber/Two-target_drive), and raw sequencing data is deposited on the European Nucleotide Archive (PRJEB80216).

Acknowledgements

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Data and code availability

All data and code supporting the findings of this study are available within the paper, its Source Data files, on Github (https://github.com/NickyFaber/Two-target_drive), and on the European Nucleotide Archive (PRJEB80216).

Author contributions

N.R.F. and J. Champer conceived the study. N.R.F. conducted the modelling. Experimental work was led by X.X., with assistance from J. Chen, S.H., J.D., and N.R.F.. Analysis was completed by N.R.F. and X.X.. J. Champer, B.A.P., J.H., and B.J.Z. provided supervision. N.R.F., X.X., and J. Champer wrote the manuscript, and all authors reviewed it.

5.6 Supplementary material

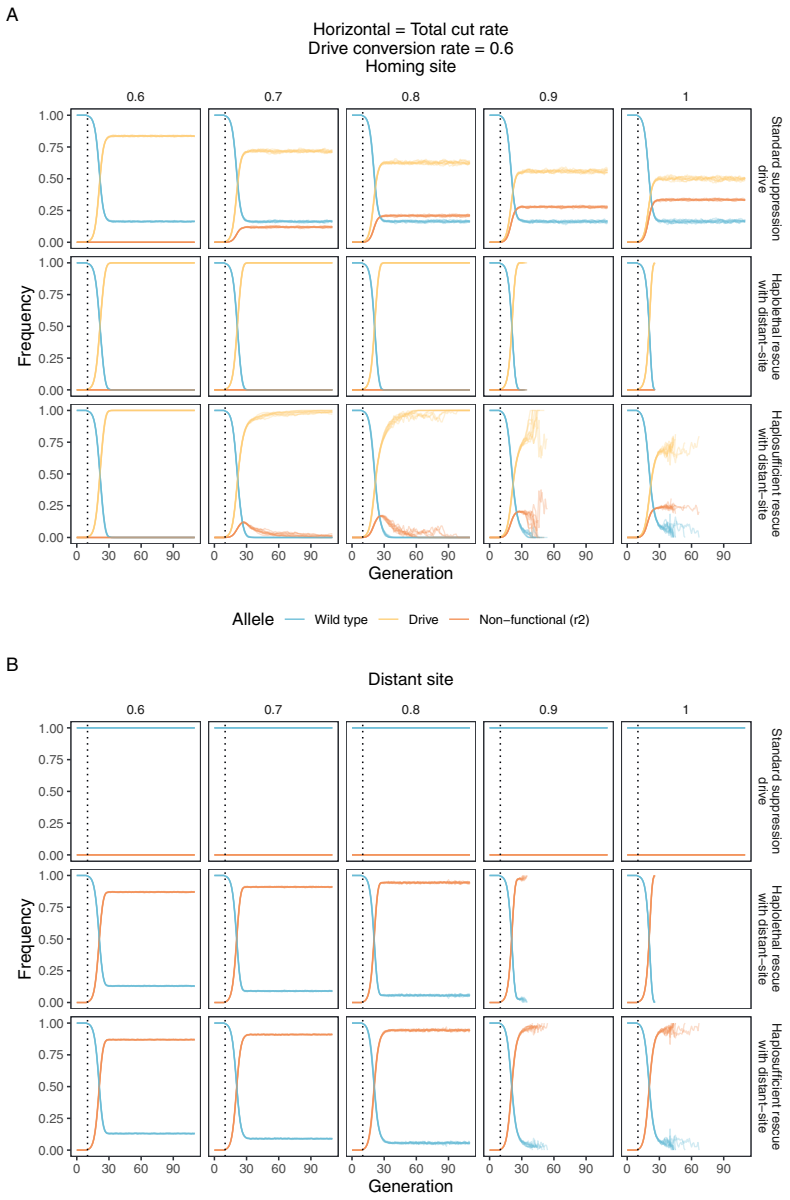


Figure S5.1. Example allele frequency trajectories. A) homing site and B) distant site allele frequencies under a conversion rate of 0.6 and varying total cut rate. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. Note that both drive conversion rate and total cut rate are absolute rates, so the drive conversion rate can never be higher than the total cut rate. For each combination of parameters, we ran 10 model repetitions for each drive.

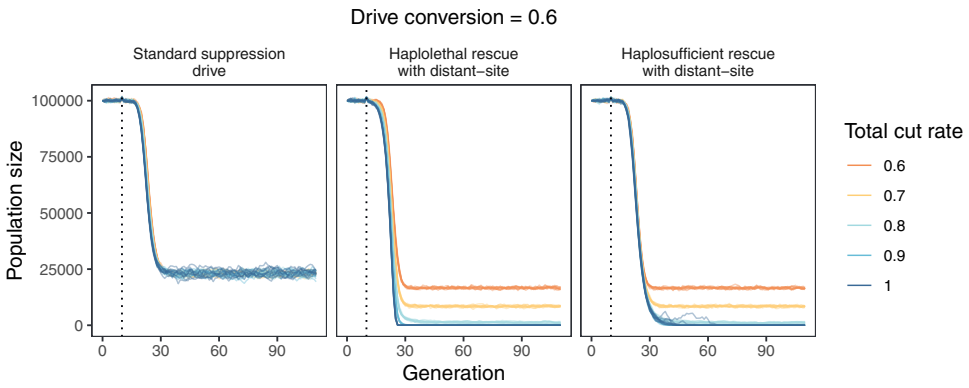


Figure S5.2. Population suppression under varying cut rates accompanying a middle (0.6) row of Figure 5.4A. Population size under a conversion rate of 0.6 and varying cut rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. Note that both drive conversion rate and total cut rate are absolute rates, so the drive conversion rate can never be higher than the total cut rate. For each combination of parameters, we ran 10 model repetitions for each drive.

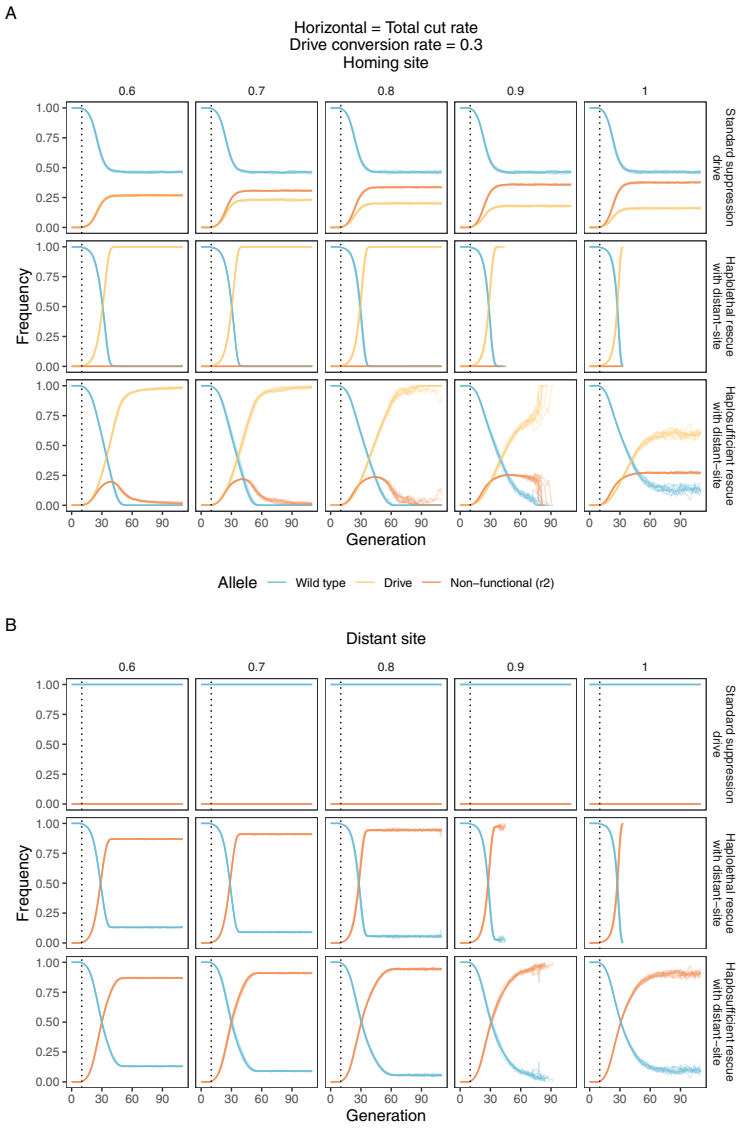


Figure S5.3. Example allele frequency trajectories accompanying a middle (0.3) row of Figure 5.4A. A) homing site and B) distant site allele frequencies under a conversion rate of 0.3 and varying total cut rate. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. Note that both drive conversion rate and total cut rate are absolute rates, so the drive conversion rate can never be higher than the total cut rate. For each combination of parameters, we ran 10 model repetitions for each drive.

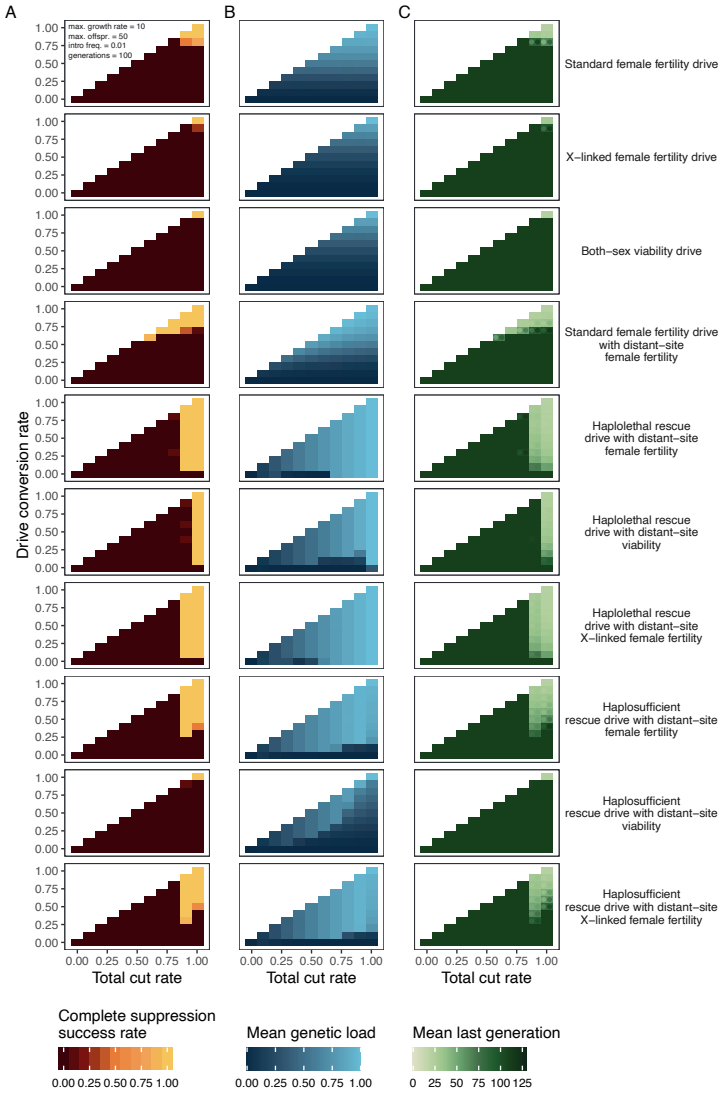


Figure S5.4. Drive performance heatmaps for additional drive variants. A) Population elimination success rate, B) mean genetic load, and C) number of generations before population elimination for all ten gene drive types under various cut and conversion rates. In C, the coloured circles in the heatmap show the 95% confidence interval around the mean. The complete suppression success rate is calculated as the number of repetitions in which complete suppression occurs divided by the total number of repetitions. Because the model stops at 111 generations, a last generation of 111 means that suppression was not successful. For each combination of parameters, we ran 10 model repetitions.

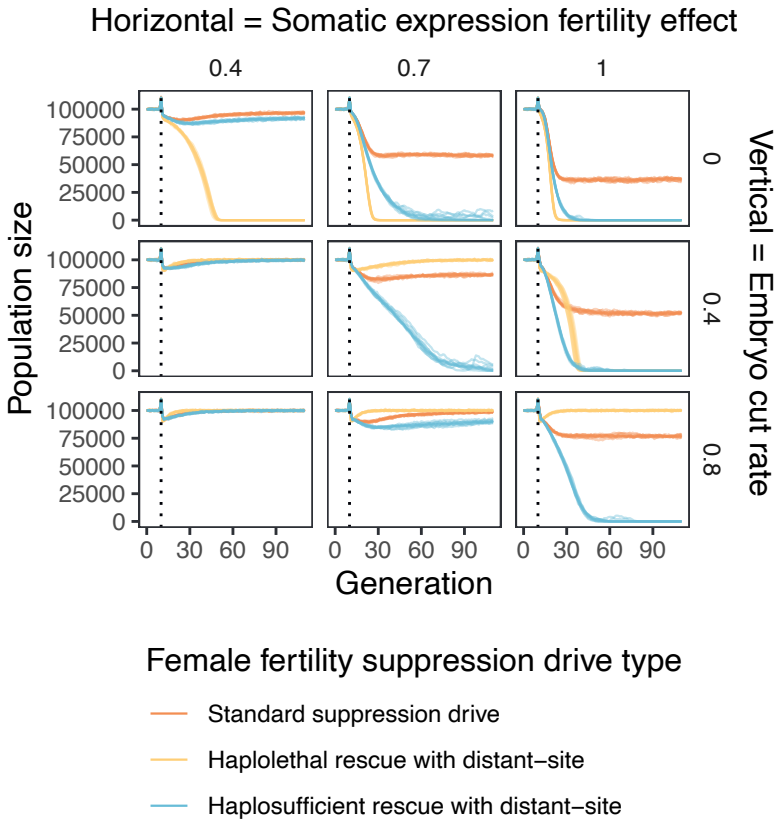


Figure S5.5. Population trajectories under variable somatic and embryonic drive activity. female fitness in drive heterozygotes from somatic expression fertility effects and variable embryo cut rates in the progeny of drive females. The gene drive is introduced after generation 10 (the dotted line), and the model is run for a further 100 generations. The introduction frequency of gene drive heterozygotes is 0.01, and the total population capacity is 100,000. The total cut rate is fixed to 0.9, and the drive conversion rate is 0.5. For the somatic expression fertility effect, 1 means there is no reduction in fertility, and 0 means females are completely sterile when the gene drive is present. For each combination of parameters, we ran 10 model repetitions.

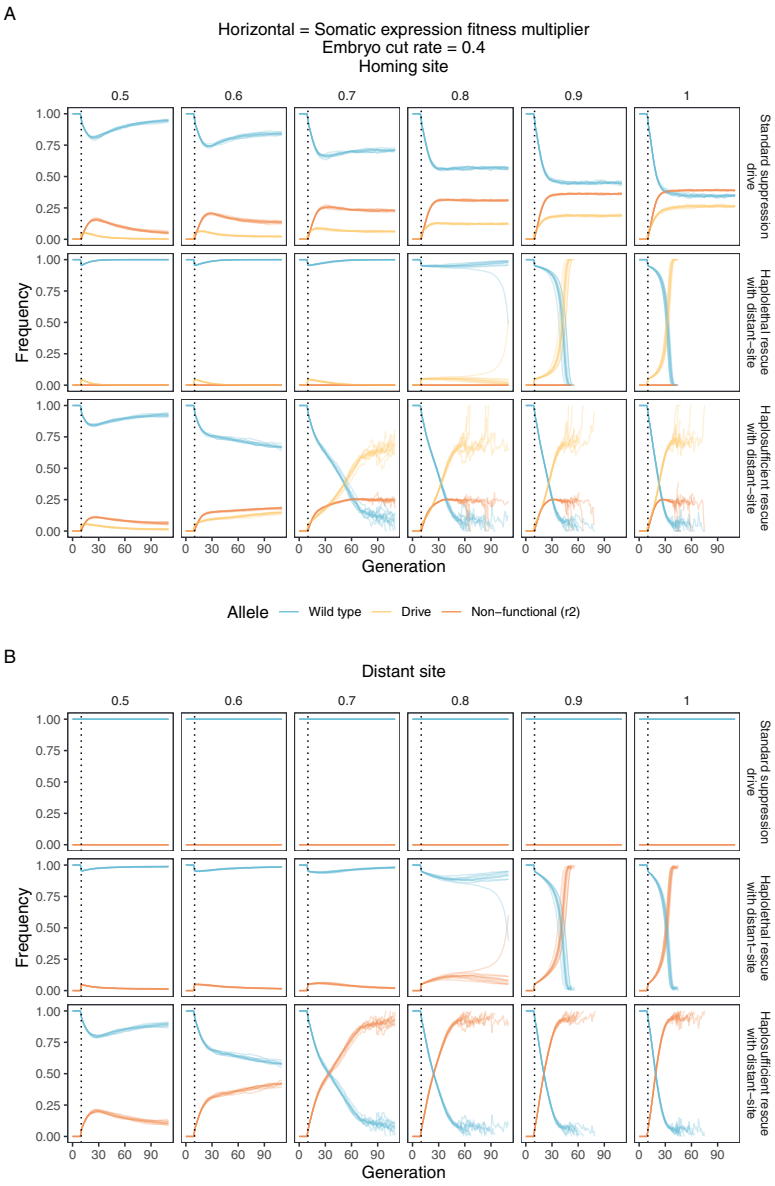


Figure S5.6. Example allele frequency trajectories accompanying a middle (0.4) row of Figure 5.5A. A) homing site and B) distant site allele frequencies under an embryo cut rate of 0.4 and varying somatic expression fitness multipliers. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. For each combination of parameters, we ran 10 model repetitions for each drive.

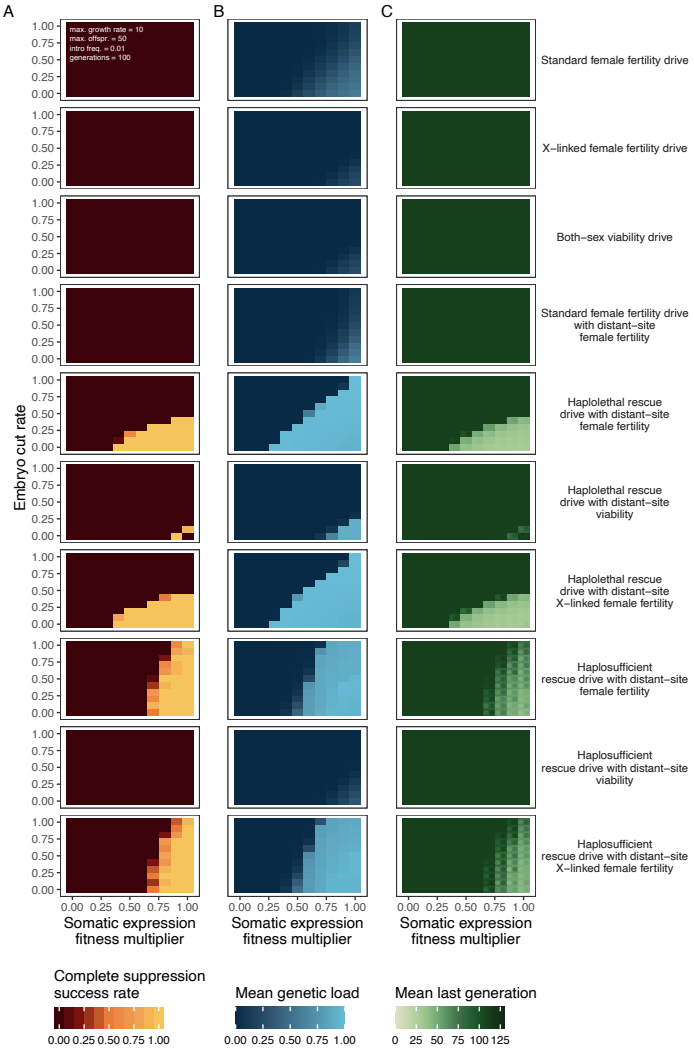


Figure S5.7. Drive performance heatmaps for additional drive variants. A) Population elimination success rate, B) mean genetic load, and C) number of generations before population elimination for all ten gene drive types under various embryo cut rates and somatic expression multipliers. In C, the coloured circles in the heatmap show the 95% confidence interval around the mean. The complete suppression success rate is calculated as the number of repetitions in which complete suppression occurs divided by the total number of repetitions. Because the model stops at 111 generations, a last generation of 111 means that suppression was not successful. For each combination of parameters, we ran 10 model repetitions.

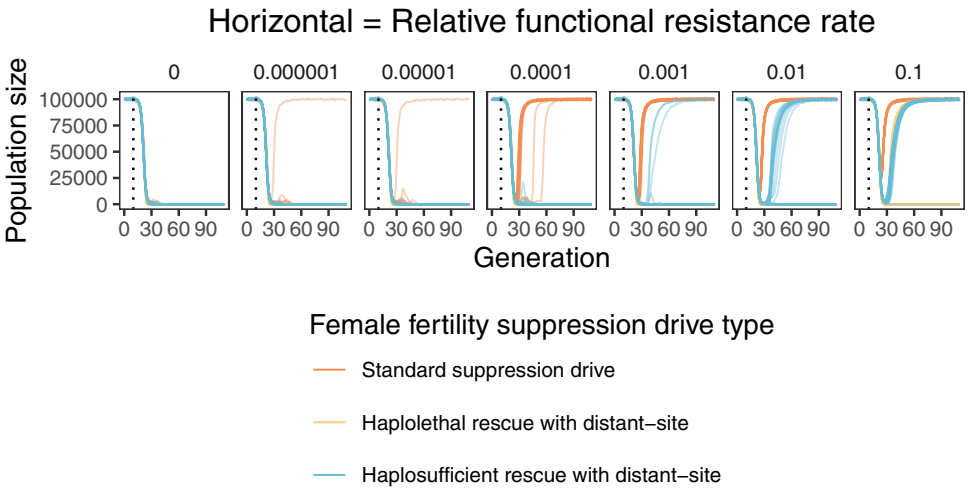


Figure S5.8. Example population trajectories under varying relative functional resistance rates. Population suppression with varying functional resistance rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. For each combination of parameters, we ran 50 model repetitions for each drive.

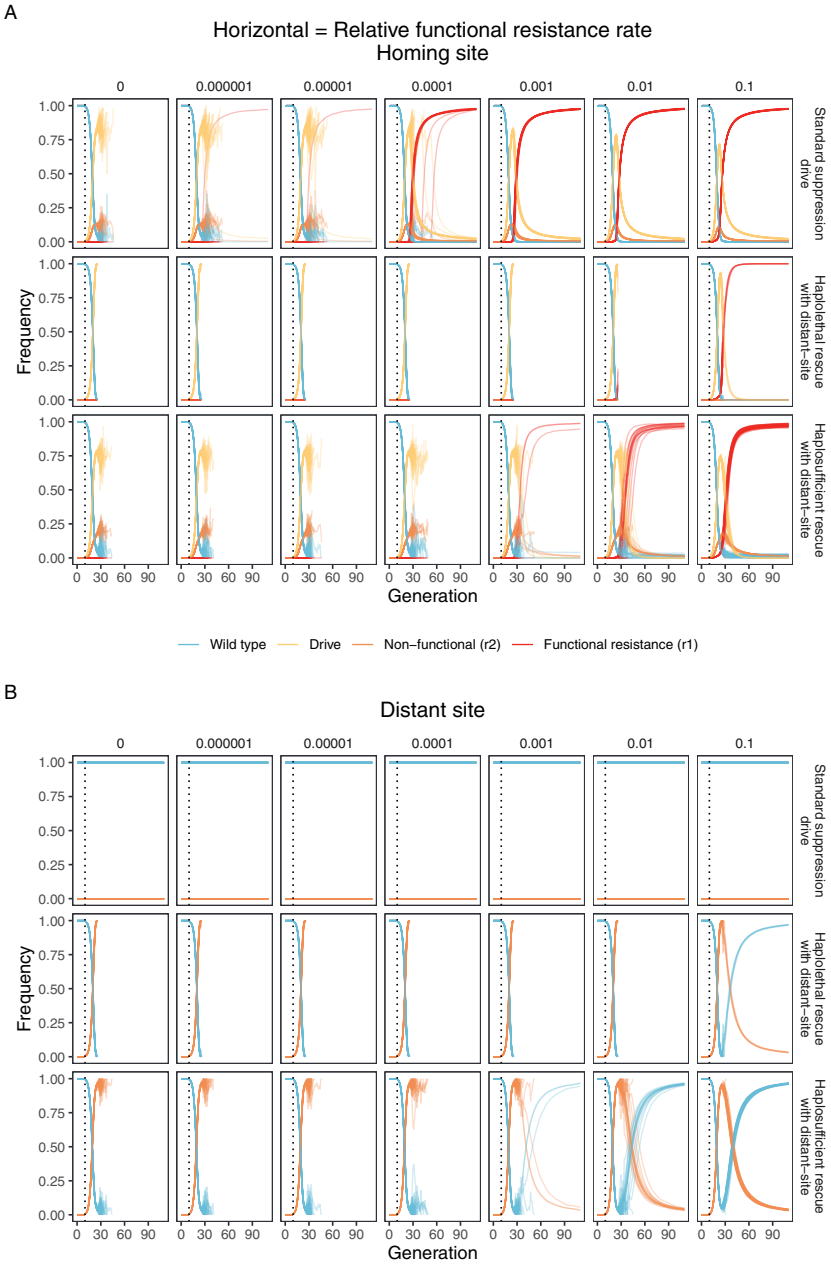


Figure S5.9. Example allele frequency trajectories. A) homing site and B) distant site allele frequencies under varying functional resistance rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. For each combination of parameters, we ran 50 model repetitions for each drive.

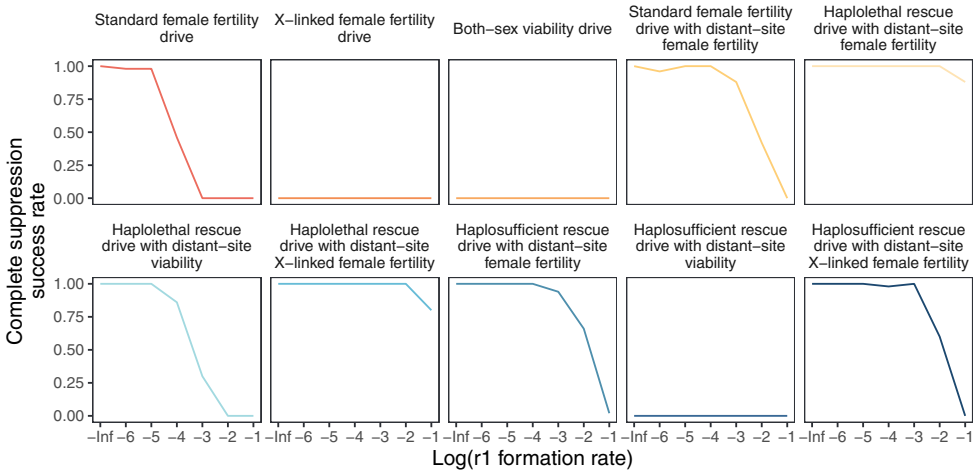


Figure S5.10. Impact of functional resistance alleles on drive performance. Complete suppression success rate under various functional resistance allele formation rates. The r1 formation rate is the relative rate of resistance alleles that are functional, rather than non-functional r2 alleles. The model is run for 100 generations after an introduction of drive heterozygotes at 0.01 frequency into a population of 100,000. We used a total cut rate 1.0, drive conversion rate 0.9, somatic expression female fertility fitness effect of 0.9, and an embryo cut rate of 0.1 in the progeny of female drive carriers. The complete suppression success rate is calculated as the number of repetitions in which complete suppression occurs divided by the total number of repetitions. Because the model stops at 111 generations, a last generation of 111 means that suppression was not successful. For each combination of parameters, we ran 50 model repetitions.

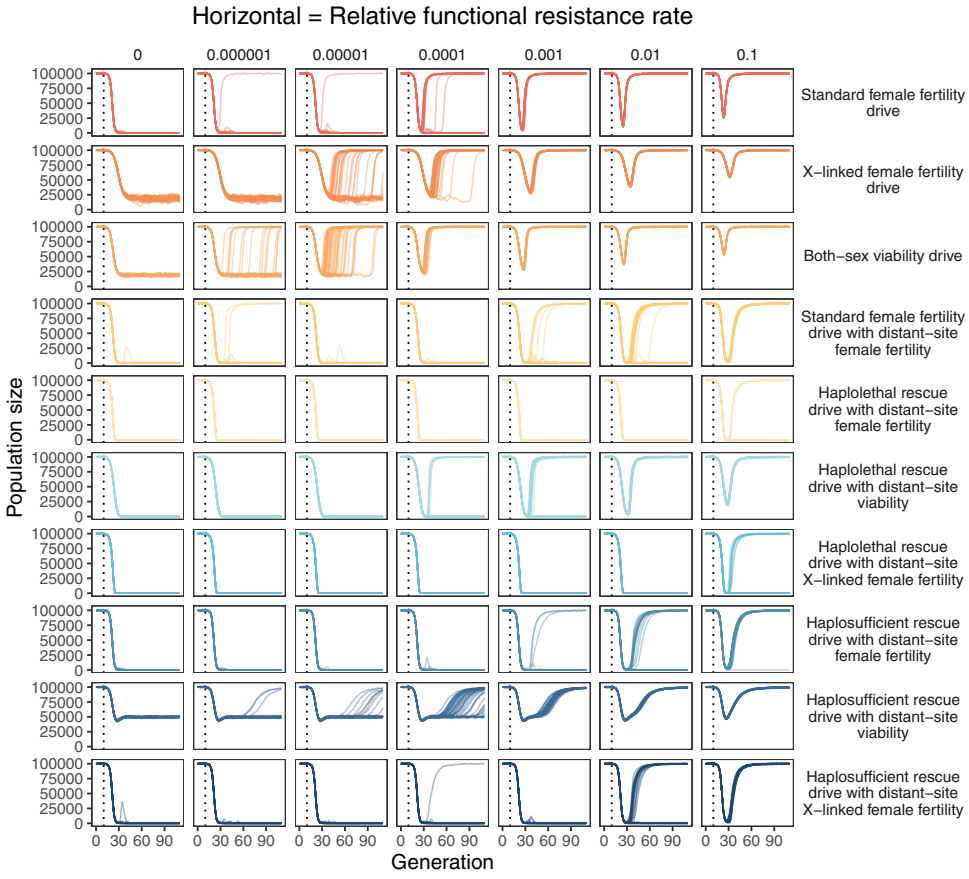


Figure S5.11. Impact of functional resistance alleles on drive performance. Population suppression with varying functional resistance rates. The r1 formation rate is the relative rate of resistance alleles that are functional, rather than non-functional r2 alleles. The model is run for 100 generations after an introduction of drive heterozygotes at 0.01 frequency into a population of 100,000. We used a total cut rate 1.0, drive conversion rate 0.9, somatic expression female fertility fitness effect of 0.9, and an embryo cut rate of 0.1 in the progeny of female drive carriers. For each combination of parameters, we ran 50 model repetitions.

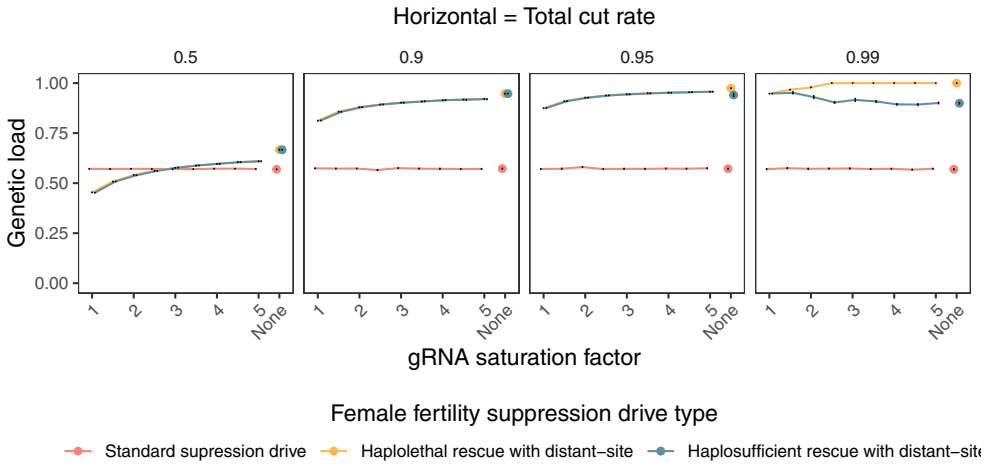


Figure S5.12. Mean genetic load with variable gRNA saturation factor and total cut rates. The gRNA saturation factor is modelled as relative Cas9 activity with unlimited gRNAs compared to the activity with a single gRNA. We assume that the distant site target gene drives have double the amount of gRNAs compared to a standard suppression drive and that the cut rates are equally reduced at both sites (the two sites are assumed to have the same number of gRNAs). The specified total cut rate is the rate for unlimited gRNA saturation factor. We used a drive conversion rate 0.5, somatic expression female fertility effect of 1, and embryo cut rate of 0. For each combination of parameters, we ran 10 model repetitions. Error bars show the confidence interval around the mean.

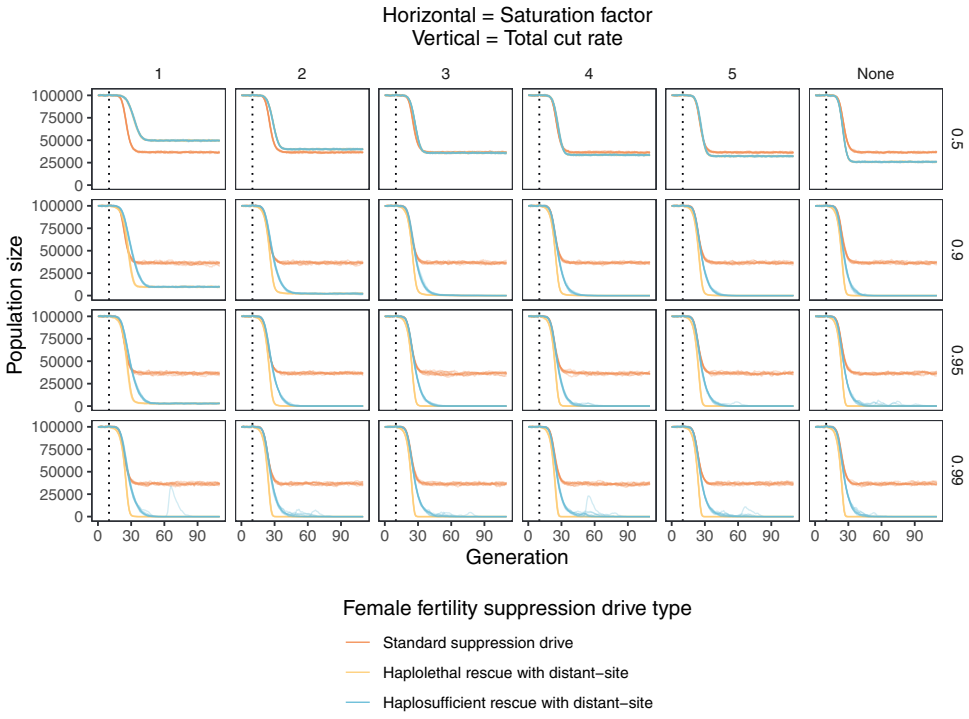


Figure S5.13. Example population trajectories under varying gRNA saturation factors and total cut rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. We used a drive conversion rate 0.9, somatic expression female fertility fitness effect of 1, and an embryo cut rate of 0 in the progeny of female drive carriers. For each combination of parameters, we ran 10 model repetitions for each drive.

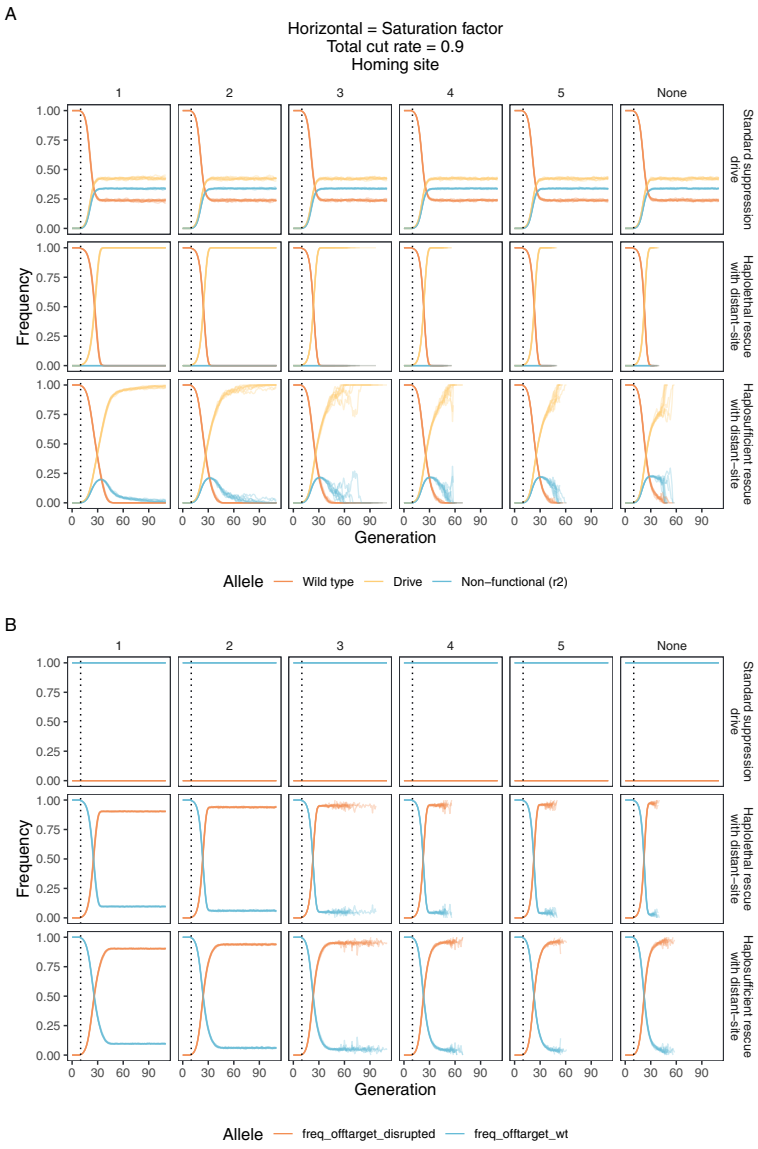


Figure S5.14. Example allele frequency trajectories. A) homing site and B) distant site allele frequencies under varying gRNA saturation and total cut rates. The gene drive is introduced after generation 10 (the dotted line). The introduction frequency of gene drive heterozygotes is 0.01, and the total population size is 100,000. We used a drive conversion rate 0.9, somatic expression female fertility fitness effect of 1, and an embryo cut rate of 0 in the progeny of female drive carriers. For each combination of parameters, we ran 10 model repetitions for each drive.

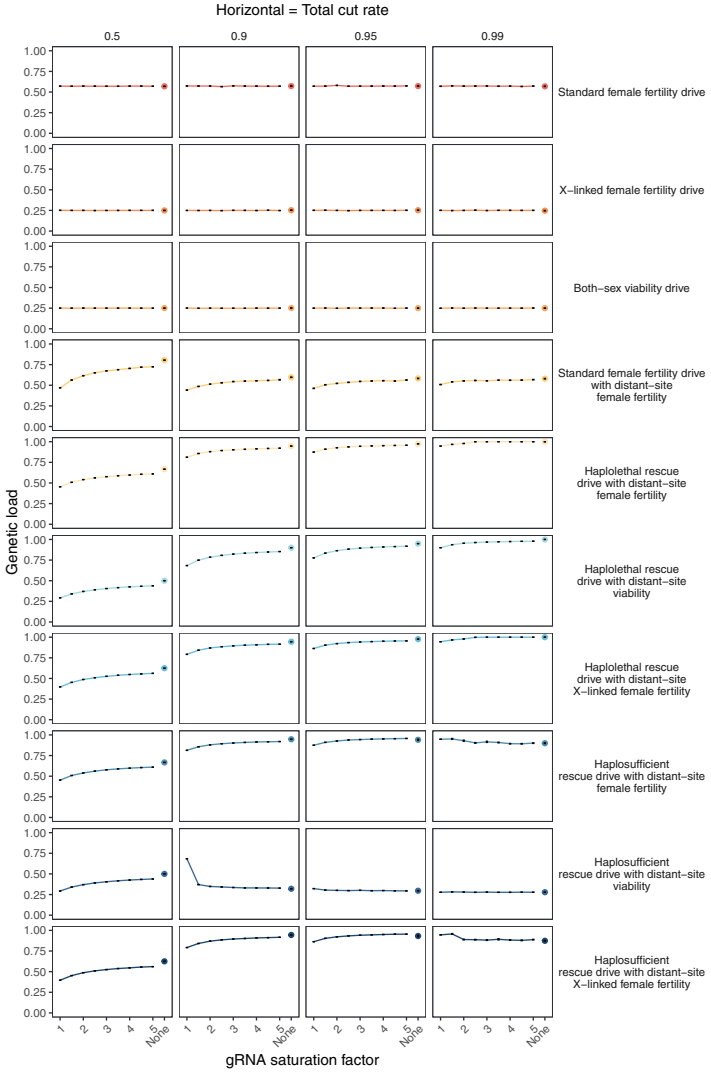


Figure S5.15. Mean genetic load with variable gRNA saturation factor and total cut rates. The gRNA saturation factor is modelled as relative Cas9 activity with unlimited gRNAs compared to the activity with a single gRNA. We assume that the distant site target gene drives have double the amount of gRNAs compared to a standard suppression drive and that the cut rates are equally reduced at both sites (the two sites are assumed to have the same number of gRNAs). The specified total cut rate is the rate for unlimited gRNA saturation factor. We used a drive conversion rate 0.9, somatic expression female fertility fitness effect of 1, and an embryo cut rate of 0 in the progeny of female drive carriers. For each combination of parameters, we ran 10 model repetitions.

Table S5.1. Embryonic resistant alleles. Sterile drive females from fertility test were randomly collected for sequencing *yellow-g* target sites.

Drive line	gRNA 1	gRNA 2	gRNA 3	gRNA 4	Number of flies with similar genotype
1A	WT	mosaic	-	-	2
1A	WT	-	mosaic	mosaic	1
1A	WT	mosaic	mosaic	mosaic	5
1A	WT	WT	WT	mosaic	1
1D	mosaic	mosaic	mosaic	mosaic	4
1D	-	-	mosaic	mosaic	1
1D	mosaic	-	mosaic	mosaic	1

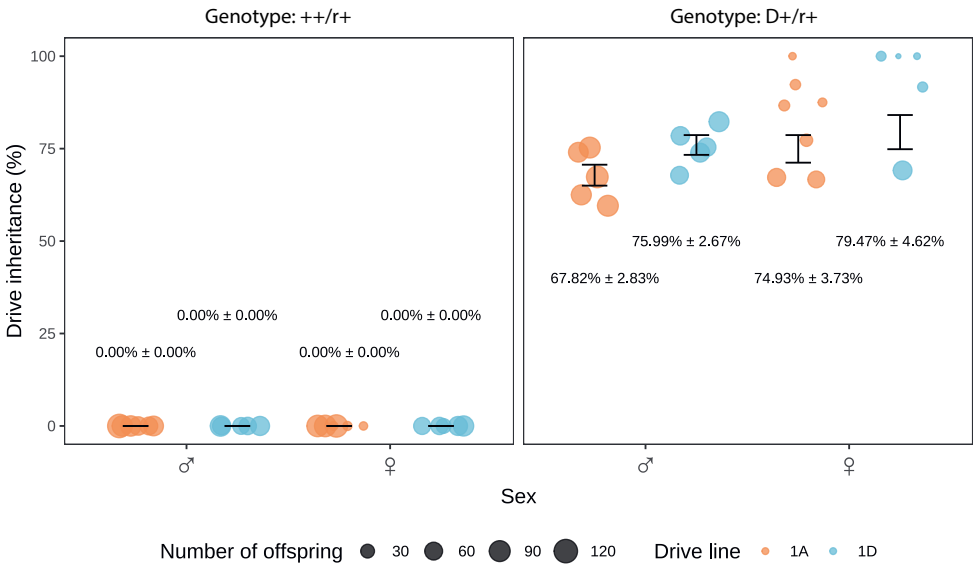


Figure S5.16. Drive inheritance rates of fertility experiment. The drive inheritance rate indicates the percentage of offspring with EGFP fluorescence from the cross between drive heterozygotes and Cas9 homozygotes with w^{1118} flies (without drive and Cas9). The size of each dot represents the total number of offspring, and whiskers indicate the standard error of the mean. For each group from left to right, n = 489, 321, 323, 296, 433, 337, 175, and 98 flies.

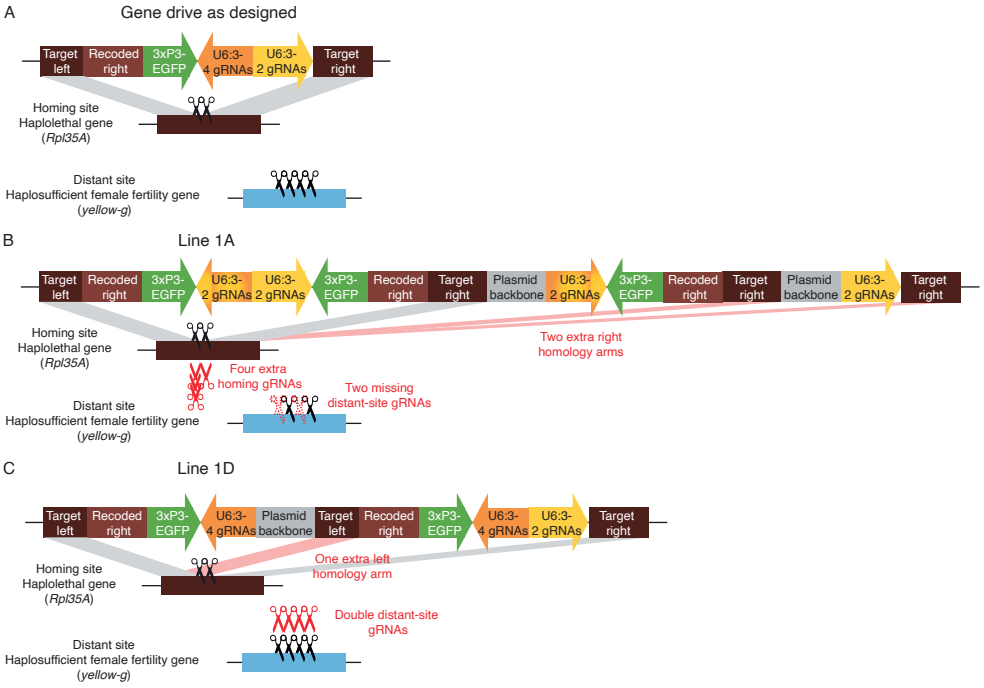


Figure S5.17. Schematic overview of the gene drive as designed and as transformed in line 1A and line 1D. A) Gene drive as designed, also shown in Figure 5.7. B) Transformation results in line 1A, and C) line 1D. For fully annotated sequences, see https://github.com/NickyFaber/Two-target_drive

"No man is an Island, intire of it selfe; every man is a piece of the Continent, a part of the maine; if a Clod bee washed away by the Sea, Europe is the lesse, as well as if a Promontorie were, as well as if a Mannor of thy friends or of thine owne were; any mans death diminishes me, because I am involved in Mankinde; And therefore never send to know for whom the bell tolls; It tolls for thee."

— John Donne, For Whom the Bell Tolls

""I'm sure," Malcolm said. "But if one of these compys were to escape from the island, to get away..."
"They can't get away"
"I know that, but just supposing one did..."
"You mean like the animal that was found on the beach?" Wu said, raising his eyebrows. "The one that bit the American girl?"
"Yes, for example."
"I don't know what the explanation for that animal is," Wu said. "But I know it can't possibly be one of ours, for two reasons.""

— Michael Crichton, Jurassic Park

Chapter 6

On safeguarding gene drive technology by targeting locally fixed alleles

This chapter is based on:

Faber, N. R., Clark, A. C., Champer, J., van den Heuvel, J., Zwaan, B. J., & Pannebakker, B. A. (2025). On safeguarding gene drive technology by targeting locally fixed alleles. *In preparation.*

6.1 Abstract

Gene drive technology may be able to aid biodiversity conservation on islands by controlling invasive species. However, due to the self-propagating nature of gene drives, they require safety mechanisms to prevent undesirable impacts on native populations. Here, we investigate the concept of the "precision gene drive". These drives target alleles that are fixed in the invasive population but absent or at low frequency in the native one. Using rodents as a relevant case study, we first demonstrate that locally fixed alleles are not likely to arise after an island invasion on timescales relevant for most invasive species (roughly 500 years). Some scenarios, such as extremely few invading individuals or very low island carrying capacity, increase drift and thus the likelihood of local allele fixation. However, their relevancy to real-world scenarios is probably low. Second, we find that adaptive evolution can lead to locally fixed alleles in relatively short time frames, but only if the selection coefficient is unrealistically high. Finally, we briefly outline and model a two-part gene drive strategy that can make use of significant shifts in allele frequency between populations, without requiring them to be fixed on the island. However, this strategy also has significant downsides in terms of practicality and robustness. We therefore conclude that overall, precision gene drives are likely not a viable strategy to ensure the safe use of gene drive technology.

6.2 Introduction

Invasive species are a major threat to biodiversity conservation and also impact human health and agriculture (Mooney et al., 2005; Pejchar & Mooney, 2009; Schaffner et al., 2013). Historically, invasive species were introduced by colonial travel (Dunlap, 1997), but nowadays, the number of invasive species continues to rise, driven by global trade and tourism (Dawson et al., 2017; Seebens et al., 2018). Island ecosystems are particularly vulnerable to species extinctions caused by invasive species due to, among other reasons, small population sizes and evolved predator naivety in island species (Fernández-Palacios et al., 2021), as their isolated evolution has left many endemic species defenceless against predation and competition. Globally, 75% of all recorded extinctions have occurred on islands, and around 50% of currently threatened species live on islands (Fernández-Palacios et al., 2021). Furthermore, because island ecosystems contain around 20% of the earth's biodiversity while only covering 7% of its land area, islands should be prioritised when conserving global biodiversity. However, for many invasive species, we lack effective and sustainable control methods, and eradication efforts are extremely labour intensive and thus usually only successful at very small scale (Keitt et al., 2011). Recently, gene drive technology has been proposed as a more powerful tool to control invasive species (Esvelt et al., 2014).

Gene drives are selfish genetic elements that bias inheritance in their own favour. After being released, a gene drive can increase in frequency and eventually reach fixation in a population. CRISPR-based homing gene drives are an especially powerful version of this technology, as they copy themselves in heterozygous carriers to replace the wild-type counterpart allele, theoretically allowing rapid spread of the drive allele (Esvelt et al., 2014). A homing gene drive can be designed to induce a fitness cost to homozygous drive carriers (it should reduce female fertility for highest efficiency) and can therefore lead to a reduction in population size or even a collapse of the entire invasive population when sufficiently efficient (Kyrou et al., 2018). However, due to the self-propagating nature of gene drives, and homing drives in particular, there are major safety and regulatory concerns. Homing gene drives can spread from the escape, or deliberate introduction, of even a few individuals, so unintended consequences are a major risk (Esvelt & Gemmell, 2017). Theoretically, the unlimited spread of a gene drive in native mainland populations could lead to the accidental eradication of those populations. The gene drive field is working to design and test

mechanisms to make a homing gene drive self-limiting (Guo et al., 2025; Min et al., 2017a, 2017b; Verkuijl et al., 2022). One proposed strategy for increased safety has been to leverage evolutionary divergence between native and invasive populations of the same species, to create a so-called "precision drive" tailored to specifically target the invasive population (Esvelt et al., 2014; Sudweeks et al., 2019). In this strategy, alleles that are fixed in the invasive population but absent or at low frequency in the native population are targeted by the gene drive; these are called locally fixed alleles or conserved private alleles. This design should ensure that a gene drive can spread through the invasive population without obstruction, but that the gene drive is largely constrained in the native population (Figure 6.1). Modelling has indicated that there is still an impact on the native population when the targeted allele is present, but the gene drive could not eradicate the native population (Sudweeks et al., 2019). However, what the upper limit for this allele frequency would be to make the magnitude of this impact acceptable in real-world scenarios is unknown and debatable (Sudweeks et al., 2019).

One may question the population genetic likelihood that locally fixed alleles arise in populations on the timescale of invasive species for gene drives to target them. In finite populations, genetic divergence is predicted to occur between invasive and native populations of a species through the evolutionary forces of genetic drift and adaptive evolution. Because a biological invasion represents a significant bottleneck and island population are usually relatively small, the effect of drift is likely to be strong (Barrett, 2015; Nei et al., 1975). Besides drift, adaptive evolution is also commonly observed on islands, and happens at a relatively fast rate. Phenotypic changes after a species migrates to an island include the evolution of gigantism, dwarfism, reduced reproductive potential, and behavioural naivete towards predators, among others (Bock et al., 2015; Prentis et al., 2008). That island-specific traits can indeed evolve rapidly is demonstrated by the invasive mice on Gough island, which have become both giant in size and show behavioural adaptation (carnivory) just 100 years after the invasion event (Gray et al., 2015; Stratton et al., 2023). Both drift and selection will most likely be based on standing genetic variation, potentially leading to significant shifts in allele frequency between native and invasive populations (Dlugosch et al., 2015; Y.-T. Lai et al., 2019). However, given the relatively short time-span since most anthropogenic invasions (±500 years) (Seebens et al., 2017), these allele frequency shifts may not be substantial enough so that alleles can be targeted by a gene drive that are high in frequency on the island and low on the mainland.

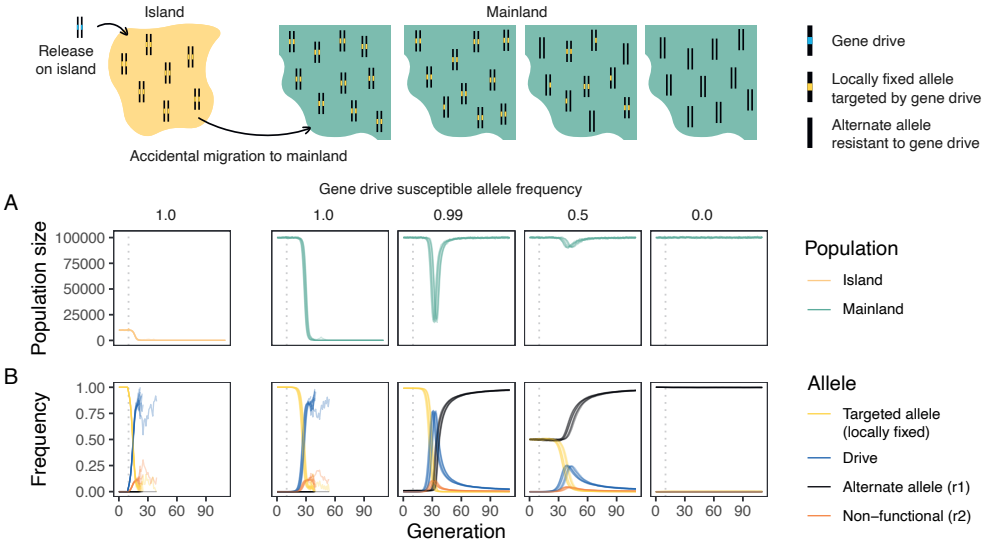


Figure 6.1. A simple demonstration of how a suppression gene drive targeting a locally fixed allele functions in theory. A) Population size and B) allele frequency trajectories after a gene drive release on the island, while including a low migration rate from island to mainland. The gene drive is released only on the island (yellow) at 0.1 heterozygote frequency, thus the drive allele frequency is 0.05. The migration rate from island to mainland is 0.001, and we assume that no migration from mainland to island occurs.

From a gene drive efficiency point of view, the feasibility of safeguarding gene drives via locally fixed alleles is also debatable. A critical factor determining the success or failure of a homing gene drive is that it can avoid the formation of resistance alleles, i.e. mutations in the genetic sequence that is being targeted by the drive (Price et al., 2020). Both pre-existing variants and resistance alleles that form through gene drive activity will thwart gene drive success. First, pre-existing variants will be exceedingly hard to find if they are at low frequencies, and would require large amounts of sampling and sequencing. Second, for most initial homing drive experiments, resistance alleles have formed within one or a few generations (DiCarlo et al., 2015; Gantz & Bier, 2015; Gantz et al., 2015; Hammond et al., 2016). So far, one of the most promising strategies to avoid the formation of resistance alleles has been to use multiple gRNAs so that resistance must form at each site individually (S. E. Champer et al., 2020; E. Yang et al., 2022). Unfortunately, this gRNA multiplexing

has been shown to reduce homing efficiency. Another strategy has been to target extremely conserved genomic sites, such as ribosomal subunit genes (J. Champer, Yang, et al., 2020) or, in insects, sex-determining genes (Kyrou et al., 2018). The need for a gene drive to target highly conserved loci is directly in conflict with the need for distinctive genetic variants to exist between populations for locally fixed alleles to form. Furthermore, resistance often still arises, albeit at very low rates (Faber, Champer, et al., 2025; Morianou et al., 2024). Fortunately, due to the flexibility of CRISPR-based genome editing, a myriad of gene drive designs have been theorised; multi-part drive designs may still be able to target variable genomic sites, as they are not as vulnerable to resistance alleles as standard homing gene drives (Faber et al., 2024; Gierus et al., 2022; Willis & Burt, 2021). These multi-part drives can separate a part of the gene drive that is needed to spread itself (targeting locally fixed alleles), from the part that induces the drive's intended effect (e.g. female sterility, targeting conserved sequences). Although resistance may form against the homing part, these alleles will only have an indirect selective advantage, and sterility can continue to be induced even when the drive is declining. At one or multiple female fertility loci, gRNA multiplexing can provide a gene drive with evolutionary stability (S. E. Champer et al., 2020; E. Yang et al., 2022). Thus, these multi-part gene drives may target locally fixed alleles while remaining evolutionarily stable.

So far, only three species have been investigated specifically for locally fixed alleles in the context of gene drive technology: mice on Australian islands (Oh et al., 2021), common wasps (*Vespula vulgaris*) in New Zealand, though only at a few genomic loci (Lester et al., 2020), and *Anopheles* mosquitoes on two islands off the African coast (Willis & Burt, 2021). Based on necessarily limited sampling, many alleles were found to be fixed in the island populations, although this number decreased when filtering for sites that could be targeted by CRISPR gRNAs and that were in female fertility genes. No alleles were found that were both fixed and entirely local; most alleles still had a considerable frequency in the native population too. These results are perhaps unsurprising in light of classic population genetic theory, which states that genetic drift is primarily related to two factors: time and effective population size N_e (Kimura & Ohta, 1969). Most rare alleles (that is, single new mutations in a population) will be lost, but a neutral allele should ultimately reach fixation in $4 * N_e$ generations on average (Kimura & Ohta, 1969). However, these numbers do not necessarily hold in the specific case of island invasions. First of all, the initial allele frequency depends on what individuals invade rather than on *de novo* mutations. Further,

Waxman (2012) shows that a sudden or gradual bottleneck in population size can decrease average time to fixation. Conversely, population expansion can increase the persistence of genetic variants and increase their average time to fixation. For beneficial mutations, their probability of fixation is similarly influenced by fluctuating population size, where N_e is determined by the harmonic average of population size over time and a determining factor together with the allele's selection coefficient (Otto & Whitlock, 1997). It is unclear how all this theory plays out in the practical case of island invasions and the formation of locally fixed alleles.

Therefore, in this study, we use a classic mainland-island population genetic model to investigate the likelihood that locally fixed alleles evolve for an invasive population on an anthropogenic time-scale, and when they do, contingent on which parameters. Ultimately, only sequencing of real-world populations can determine the possibility of targeting locally fixed alleles with a precision drive, but the results of our study temper expectations of targeting locally fixed alleles as a safeguarding strategy for the containment of gene drives. We show that especially for islands originally invaded by a large number of individuals, islands supporting larger numbers of individuals, or populations for which recurring introductions are likely ongoing, the formation of locally fixed alleles within ~500 years is highly unlikely. We conclude that using a strategy for which the safeguarding is built into the drive design (e.g. daisy-drives) is more feasible, rather than relying on the presence of locally fixed alleles.

6.3 Methods

6.3.1 Island invasion model

We built a population genetic model using the software SLiM (version 5.1.0) in combination with R (version 4.5.0) to simulate the evolution of allele frequencies after an island invasion (Haller et al., 2025; R Core Team, 2025). Our model consists of a mainland population and an island population, both of varying carrying capacities, and with a certain amount of variance over the years (Figure 6.2). To keep our model as general as possible while mimicking rodent population size dynamics, we use a Wright-Fisher model with some variance and spikes in population size, typical for rodent populations in the wild (Andreassen et al., 2021). We chose to model rodents because they include mice and rats,

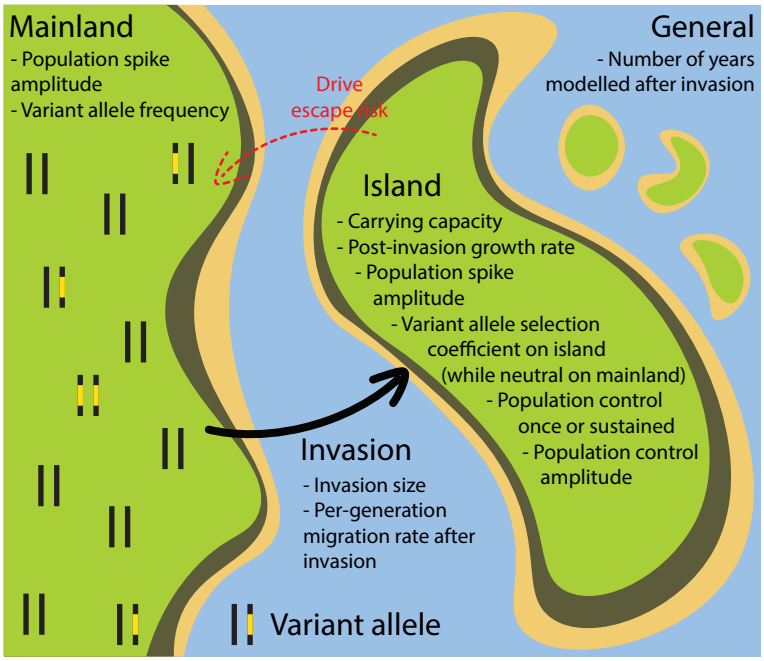


Figure 6.2. A schematic overview of our population genetic model and varied parameters.

which are among the world's most impactful invasive species, and they are present on many islands (Luque et al., 2014; Simberloff, 2009). Specifically, our model includes spikes in population size on average every five years in both the mainland and island populations (but not necessarily in the same years). We have added stochasticity to spike amplitude so the standard deviation equals half the spike amplitude. When we drew a negative number for spike amplitude, we redrew until the value was positive.

Starting in the mainland population (100,000 individuals), a single genetic variant locus is simulated with two alleles, which are initiated at a range of frequencies (0.01 - 0.99). We model an invasion of a set number of individuals to the island (50 by default), after which this island population proceeds to grow to the island's carrying capacity (10,000 by default) with logistical growth under a certain growth rate (1.2 by default). Furthermore, on the island, we model the potential switch from a neutral mutation to a deleterious or beneficial one with varying selection coefficients (-0.1 - 0.1). We also model optional continued migration to the island after the initial invasion at a certain rate per year (determining what frac-

tion of the island population came from migration from the mainland population, 0 by default). At year 500, we have added the option for population control of varying amplitude (0.5 - 0.99), which can either be a one-time intervention (similar to the unsuccessful eradication attempt of the invasive mice on Gough island, Samaniego et al., 2022), or sustained population control until the end of the simulation. In general, we simulated 1000 years after the island invasion, which is significantly longer than the time passed after known, anthropogenic invasion events, but still modest on an evolutionary timescale.

We track the frequency of a single variant allele over time in both populations to ultimately calculate the absolute difference between mainland and island allele frequency in the final year. For every simulation, we run 100 replicates. Although we could have run many more replicates to approximate the total number of variant sites in a genome, only a handful of genes are likely to be viable targets for a suppression gene drive (Clark et al., 2024). An overview of parameters, which we have chosen to remain as close to ecologically relevant as possible, is shown in Figure 6.2. For the full model code, see our GitHub (https://github.com/NickyFaber/LocallyFixedAlleles).

6.3.2 Gene drive targeting locally fixed alleles model

The model used for Figure 6.1 and for our two-target drive with self-destruct in Figure 6.8 are adapted from a previously existing two-target gene drive model (Faber et al., 2024). For code and details, also see our GitHub (https://github.com/NickyFaber/LocallyFixedAlleles)

6.4 Results and discussion

6.4.1 Fixation of alleles under neutral drift

For our default model conditions, we plotted population size, allele frequency and allele frequency differences between mainland and island (Figure 6.3). We model a mainland population of 100,000 individuals and an island carrying capacity of 10,000 individuals. We include spikes in population size every five years in which population size doubled on average to mimic rodent populations (Figure 6.3A). For the invasion to the island, we randomly drew 50 individuals from the mainland. Thereafter, the island population grew to a default carrying capacity of 10,000 individuals under a growth rate equal to 1.2, meaning

that the population can grow by 20% each year. Alleles were neutral in this simulation and only changed in frequency due to drift.

We saw that, as expected, there was more genetic drift in the island population than in the 10-fold larger mainland population (Figure 6.3B). We saw that both the initial bottleneck and the following years was when the biggest shift in allele frequencies occurred on the island, after which allele frequencies remained more or less stable. Differences in allele frequency were initially low, but do increase over the years (Figure 6.3C and D). After 10,000 years, many alleles had fixed on the island, while at the same time, there was barely any drift on the mainland. This result, while expected, marks an important fact: even with a significant amount of drift on the island, the difference with the mainland was still constrained by the initial allele frequency on the mainland. For example, with an initial allele frequency of 0.5 on the mainland, where almost no drift occurred due to the large population size, even complete fixation on the island would only lead to a maximum allele frequency difference around 0.5. When the initial allele frequency on the mainland was low, drift on the island was much more likely to drift to zero than to fixation, and *vice versa* when the initial allele frequency was high. Therefore, an ideal locally fixed allele that is absent on the mainland (also called a conserved private allele) appeared highly unlikely to form due to genetic drift alone.

Overall, almost all frequency differences ranged between 0 and 0.5, and in the majority of replicates, alleles did not reach a frequency difference over 0.25 even after 10,000 years. From these results, we conclude that the ±500 years, which is the upper limit for how long most invasive species have been present on islands (Seebens et al., 2017), provide very little time for significant shifts in allele frequencies due to genetic drift, at least under our parameter settings.

6.4.2 Varying invasion and island parameters

Next, we varied a range of parameters in our simulation to determine under what conditions we could feasibly expect to find alleles that have become more differentiated in frequency between mainland and island populations. From here on, we focus on divergence at 1000 years after invasion, and show frequency dynamics stemming from an initial 0.1 mainland frequency in our main plot, as intermediate allele frequencies are usually rare in wild populations as is expected in neutral evolution models (Kimura, 1979).

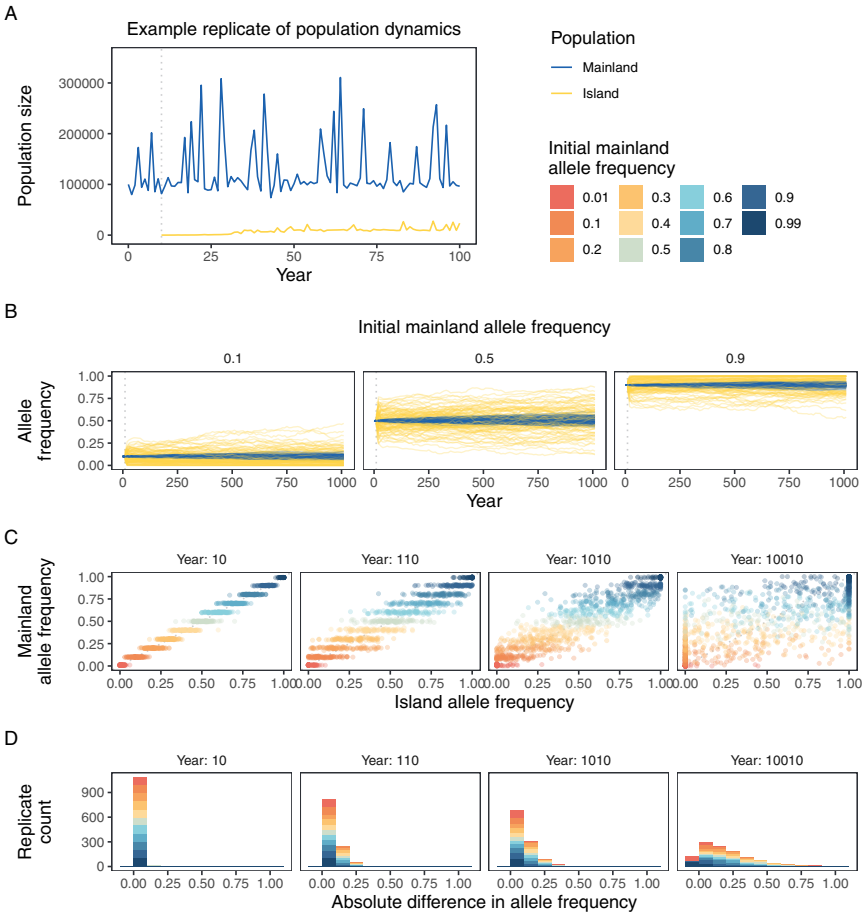


Figure 6.3. Neutral alleles drifting in our default model. A) Example replicate of population size dynamics. Note that for population size, only 100 years are shown. B) Allele frequency trajectories over 1000 years. Data only shown for initial mainland allele frequencies 0.1, 0.5, and 0.9, while panels C and D contain the full frequency range (0.01 - 0.99). C) Allele frequency in the island population plotted against allele frequency in the mainland population in generations 10 (when the invasion occurs in the model), 110, 1010, and 10010 (the default time that we model hereafter is only 1010 years). The dotted diagonal indicates equal allele frequencies between the populations. D) Stacked histogram of absolute differences in allele frequency between the mainland and island populations in the same years as in C. Note that the bar left of 0.0 represents only exact 0.0 values and the bar right of 1.0 represents only exact 1.0 values to distinguish small differences from no differences.

6.4 Results and discussion

We varied six different parameters of our model to represent ecologically relevant scenarios (Figure 6.4 and Figure 6.5): invasion size, island baseline carrying capacity, population growth rate after invasion, ongoing migration from mainland to island, population control after 500 years (amplitude of control and whether one-time or sustained until the simulation end), and mainland and island population spike amplitude.

For the number of invading individuals (tested at 5, 50, and 500 individuals), a very small number was necessary to obtain significant founder effects, as indicated by the spread in island allele frequencies on the dotted line of generation 10 (Figure 6.4A). When only five individuals invaded the island, founder effects are logically much more significant than when 50 do. When 500 individuals invaded, this group was a near-perfect representation of the mainland population. Overall, only when five individuals invaded did allele frequencies differ up to around 0.5 (Figure 6.4B). Although such small invasion sizes are not unheard of, for example the single fertilised female invasion of the Asian hornet to Europe (Arca et al., 2015), most mammalian predator species were likely either introduced in larger sizes, whether accidentally or on purpose (Pichlmueller et al., 2020). Worse yet, some species have also been introduced multiple times from different genetic source populations, thus leading to increased genetic diversity rather than founder effects on the island (Oh et al., 2021). Although it is hard to estimate exactly how many individuals invaded an island hundreds of years ago, we believe these numbers to represent a significant limitation on the formation of locally fixed alleles due to founder effects, as invasions are likely to be in the range of tens to hundreds of individuals in many cases (Alves et al., 2022; Aplin et al., 2011; David-Gray et al., 1998; Pichlmueller et al., 2020; Searle et al., 2009), although individuals may be genetically more similar to each other if from the same source population (Alves et al., 2022).

We next varied island carrying capacity (tested at 1,000, 10,000, and 100,000, Figure 6.4C). Only the 1,000-individual population led to considerable amounts of genetic drift (Figure 6.4D). Similar to invasion size, it is hard to determine how many mice inhabit a given island. In general, rodent population size is highly variable across seasons and environments. For mice in New Zealand, area densities can be as high as 46 individuals per hectare (Wilson et al., 2018), but they also exist at lower densities of 0.02 - 1.8 individuals per hectare (Wilson & Lee, 2010). Similarly, rats in New Zealand can occur at densities up to 36 individuals per hectare under resource-abundant conditions (Harper & Rutherford, 2016) and as low as

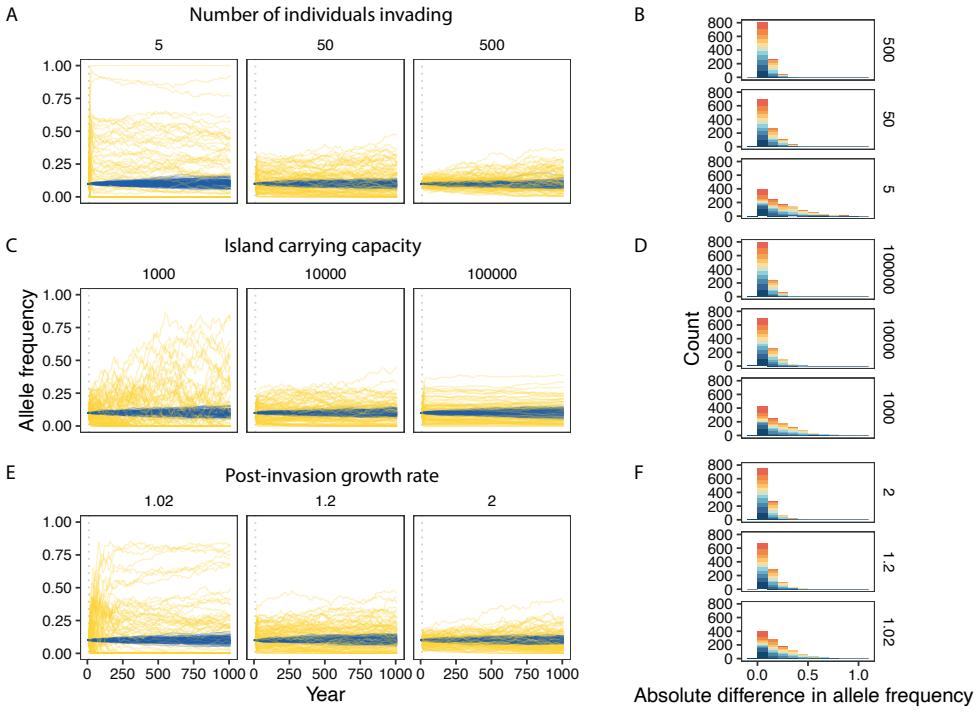


Figure 6.4. The effect of model parameters on the amount of drift in the island population, part I. A, C, E) Allele frequency trajectories over 1000 years after invasion while varying a single model parameter (the middle column being the default). B, D, F) Absolute difference in allele frequency between the mainland and island population. A single parameter was varied from default settings (Figure 6.3): A & B, number of individuals invading the island, C & D, the carrying capacity of the island, and E & F, the growth rate of the island population after the initial invasion. For colour legends, see Figure 6.3.

0.5 individuals per hectare as well (Christie et al., 2015). Conventional control methods can feasibly eradicate invasive populations on islands up to 500 ha, or even as large as 20,000 ha (Keitt et al., 2011). Therefore, most islands for which gene drives might be valuable tools will support many more small rodents than simulated here. The census population size may not be equal to the effective population size, however, with the latter usually being considerably lower and leading to increased drift (Palstra & Ruzzante, 2008). Even so, for example on an island such as Gough with an area around 6500 ha, the effective population size of mice is estimated to be around 10,000 (Gray et al., 2014). Thus, large population

size is another factor that likely limits genetic drift in island populations.

The smaller the post-invasion growth rate of the island population (tested at 1.02, 1.2, and 2), the more drift occurred (Figure 6.4E). As the population size remained smaller for longer, more drift could occur in the initial years after invasion, leading to larger differences in allele frequency between island and mainland. For many exotic plant species, such a lag period after invasion is common (Aikio et al., 2010). It is also known to occur for some invasive animals, for example, certain exotic bird species on the Hawaiian islands remained low in number for years (Aagaard & Lockwood, 2014). Similarly, the grey squirrel was established as an exotic animal in the United Kingdom for decades exclusively in the London area before their rapid expansion to the entire United Kingdom in recent decades (Middleton, 1930). Usually, some change in the environment or population itself suddenly enables population growth. For invasive plants, this change may be the clearing of native forest or destruction of native vegetation by invasive animals (Cheke, 1987). For animals, this change may be increased dispersal due to urbanisation and increased human mobility (Spear et al., 2013). For most of the highly problematic invasive species such as rats and mice, however, growth rates are usually immediately high, as palatable native vegetation is highly abundant right away. The initially extremely high growth rates are often followed by a steep decline and ultimately an equilibrium afterwards around the carrying capacity (Cheke, 1987). Overall, much is unknown about these lag phases, and their lengths are often hard to predict for any given exotic species (Coutts et al., 2018). For most species, however, the lag phase will be more akin to several decades rather than centuries, so our modelled scenario with a growth rate of 1.02 is likely unrealistically low for most species.

The more migration to the island continuously occurred (tested at rates 0, 0.001, and 0.01), the more similar the populations remained in allele frequency (Figure 6.5A). For many islands, there is evidence of multiple invasions (Pichlmueller et al., 2020), if not a constant migration rate. Again, although it is hard to map rodent migration, it is generally accepted that all ships had rats on them until at least the 1930s (Skotnes-Brown, 2023), and likely even to a limited degree up until now (Rubbo, 2022). Therefore, for some species, a constant (though low) migration rate to the island may be realistic. In our model, a migration rate of 0.001 means that 10 individuals made it to the island on a yearly basis. This rate was already enough to reduce the already low amounts of drift to near-zero, let alone a migration rate of 0.01, meaning 100 individuals made it to the island every year (Figure 6.5B). For

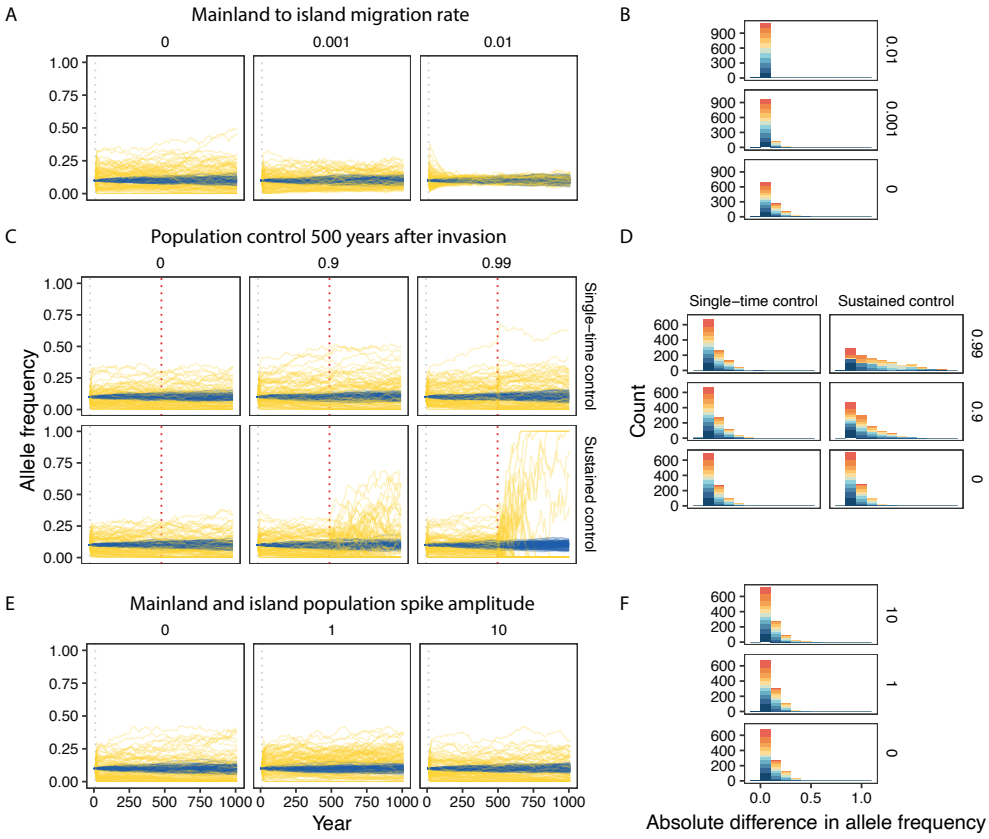


Figure 6.5. The effect of model parameters on the amount of drift in the island population, part II. A, C, E) Allele frequency trajectories over 1000 years after invasion while varying a single model parameter (the left column being the default except in E, where the middle plot is the default). B, D, F) Absolute difference in allele frequency between the mainland and island population. A single parameter was varied from default settings (Figure 6.3): A & B, the mainland to island migration rate, C & D, the control of the invasive population through conventional methods, both single-time and sustained, and E & F, the amplitude of population spikes on both the mainland and the island. For colour legends, see Figure 6.3.

a gene drive or any type of population control to achieve lasting eradication, the migration rate to the island must be kept at zero, especially after eradication is complete. For this reason alone, limiting any migration is a main priority for the potential of gene drives to

aid conservation. This is an extremely hard task, however, especially when islands are close to other landmasses (Pichlmueller et al., 2020). Some larger islands such as New Zealand, Australia, Hawai'i, and the Galapagos islands have extremely strict biosecurity controls at their borders to prevent incoming migration. Even so, species make it through occasionally, as illustrated by the recent discovery of yellow-legged hornets in New Zealand (Lester, 2025). Interestingly, in the case of gene drive deployment on islands, the outbound migration of an established invasive species to the mainland is also important, but currently largely unknown.

Conventional population control (tested at rates 0, 0.9, and 0.99 for both sustained and one-time control) may aid the formation of locally fixed alleles (Figure 6.5C). A single-time control in year 500, similar to the unsuccessful eradication effort of mice on Gough island (Samaniego et al., 2022), did not have a considerable impact on allele frequency trajectories. However, especially sustained population control had a positive impact on drift through severely reducing the island carrying capacity (Figure 6.5D). As the efficiency of gene drives in mice has so far been limited (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021), it has been suggested that they may nevertheless serve as a valuable complementary tool alongside existing control strategies to eliminate the last remaining individuals (Birand et al., 2025). However, it is already clear that conventional control methods are constrained by both scale and accessibility of target areas, so conventional population control that reduces the population by 99% over large areas will be exceedingly hard to achieve.

Whether or not a population experienced occasional spikes in size (tested for amplitudes 0, 1, and 10) did not seem to impact genetic drift (Figure 6.5E and F). Otto and Whitlock (1997) have shown that the harmonic mean of population size determines allele fixation probability, indicating that outlier years with high population size are much less important than average population sizes, and that very low outliers are disproportionally important. Thus, the lower bound for population size (bottlenecks) are more important than population spikes, which likely only proportionally amplify and decrease existing allele frequencies.

6.4.3 Differentiation due to adaptive evolution

While genetic drift was highly unlikely to provide enough cause for considerable genetic differentiation between island and mainland populations, perhaps adaptive evolu-

tion would be more likely to achieve this differentiation. As mentioned, island populations can rapidly evolve to adapt to their new environments as demonstrated by the giant carnivorous mice of Gough island, who have only been there for around 100 years (Gray et al., 2015; Stratton et al., 2023). At the same time, body size is an extremely polygenic trait (Yengo et al., 2022), and behaviour has notoriously low heritabilities in the first place (Roff & Mousseau, 1987). Therefore, the selection on individual alleles is likely to be small. We showed that for an allele frequency to shift significantly between populations on an anthropogenic time-scale (±500 years), extremely large selection coefficients were necessary (Figure 6.6A and B). Therefore, we conclude that, unless there is strong selection for a specific (monogenic) trait on an island, adaptive evolution likely only leads to locally fixed alleles over longer time-scales than relevant for controlling invasions that are damaging to local biodiversity.

6.4.4 A multi-part gene drive safeguarding strategy through a built-in self-destruct mechanism

Taking our results in combination with previous studies that have looked for locally fixed alleles in real invasive populations (Lester et al., 2020; Oh et al., 2021; Willis & Burt, 2021), we conclude that alleles that are both fixed on islands and completely local (that is, absent on the mainland) are highly unlikely to exist. Fixation may still be possible on islands, but as discussed before, this is both hard to be sure of and, in a classical precision gene drive design, can still lead to unacceptable impacts on mainland populations (Figure 6.1). Therefore, perhaps the only feasible way to target locally fixed alleles is to use a multi-part gene drive strategy (Willis & Burt, 2021). These multi-part drives separate a part of the gene drive necessary for spread (targeting a locally fixed allele), from the part that induces the drive's intended effect (targeting conserved sequences). Below, we detail an additional potentially useful design that can function based on a shift in allele frequency rather than fixation by targeting locally extinct alleles with a self-destruct mechanism.

Instead of a precision drive targeting a locally fixed allele, we propose that a gene drive could be designed so that it targets a highly conserved sites with multiplexed gRNAs (that is, in the most robust manner currently known, E. Yang et al., 2022), but with one extra component that acts as a self-destruct mechanism: one or multiple additional gRNAs that target a locally extinct allele (LEA) (this is still a locally fixed allele, only the alternative allele

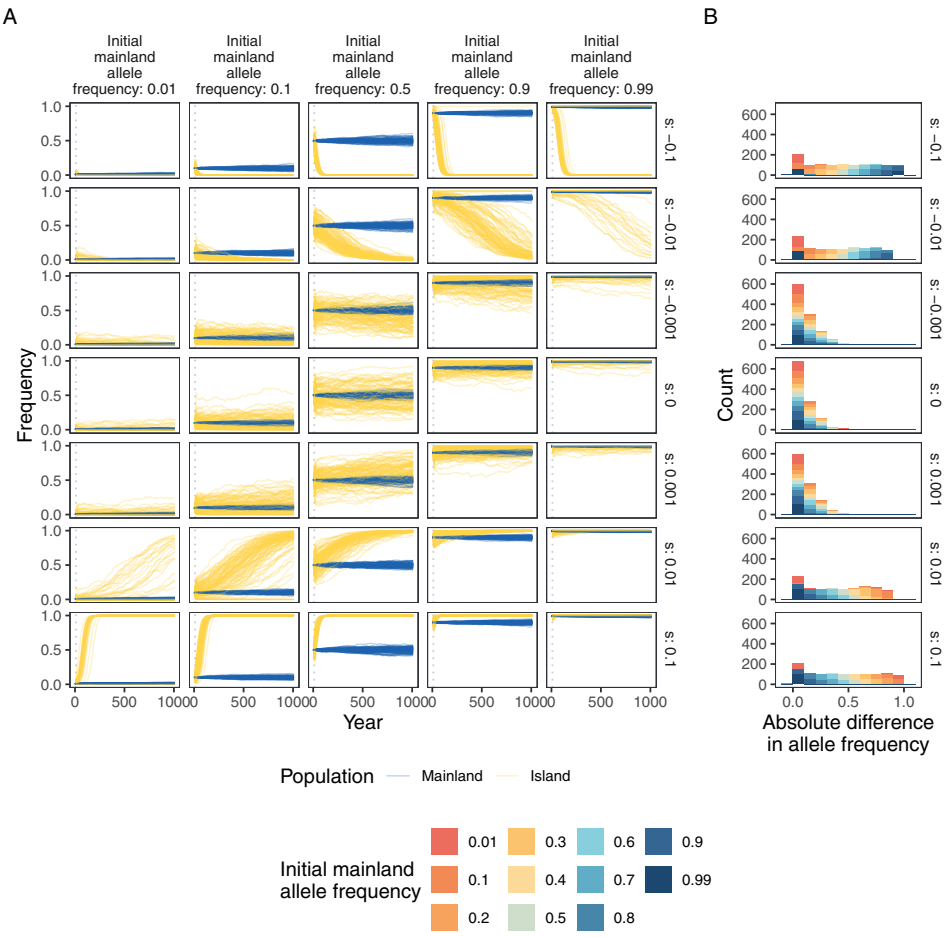


Figure 6.6. The effect of adaptive evolution of the invasive island population. A) Allele frequency trajectories over 1000 years after invasion under various selection coefficients (s) and initial mainland allele frequencies, and B) the resulting absolute difference in allele frequency between the mainland and island population. Data only shown for initial mainland allele frequencies 0.01, 0.1, 0.5, 0.9, and 0.99.

is targeted). This allele should be in a haplolethal gene or another haploinsufficient gene with a high dominant fitness cost (Figure 6.7), which, although still somewhat restrictive as haplolethal genes may have fewer genetic variants than less essential genes, could provide more potential gene targets than a gene drive targeting female fertility currently has (Clark

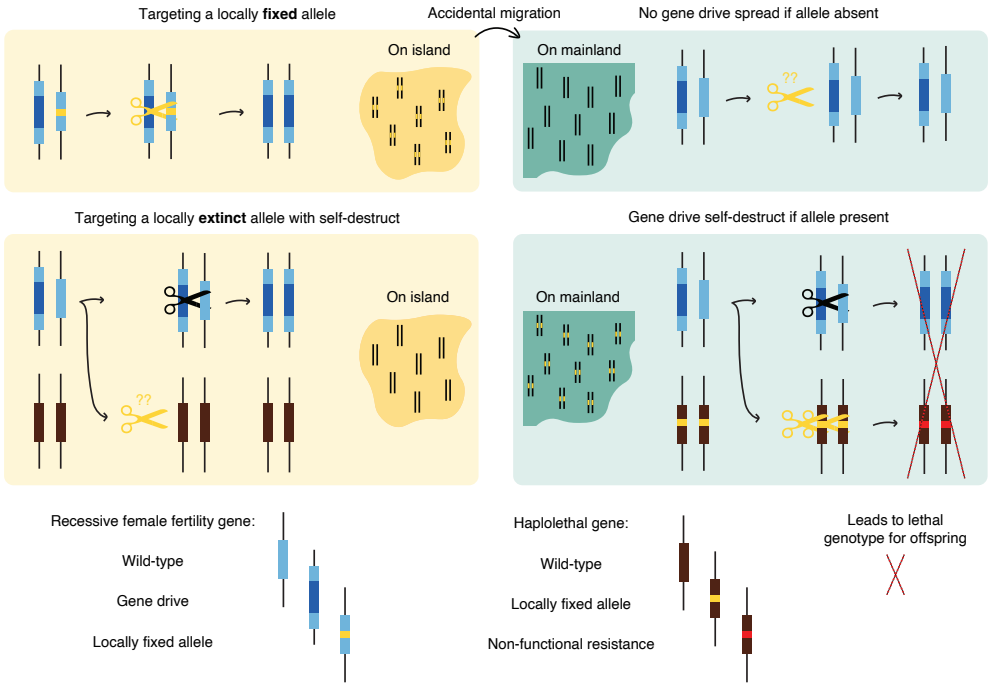


Figure 6.7. Schematic of a gene drive that targets a locally extinct allele (LEA) at a distant site with self-destruct. As opposed to a gene drive that targets a locally fixed allele to spread, this strategy is less vulnerable to the allele not being entire fixed or extinct in either population.

et al., 2024). As the self-destruct target will mostly be absent on the island, the gene drive will function as intended. Should gene drive individuals escape to the mainland, however, they will usually cross with an individual carrying the susceptible self-destruct allele, thus, subsequently, the gene drive will disrupt it and cause non-viability, thereby removing itself. Should susceptible alleles still be present at low frequency in the island population, the gene drive will only lose some efficiency while still removing those alleles. Therefore, this gene drive design can provide local dynamics towards elimination of the invasive species, based on only a difference in allele frequency (or perhaps even a significant difference in population size), without having to rely on complete fixation or complete privateness.

We have used a simple two-locus population genetic model to briefly investigate the dynamics of this system (Figure 6.8). We modelled a mainland and an island population, including

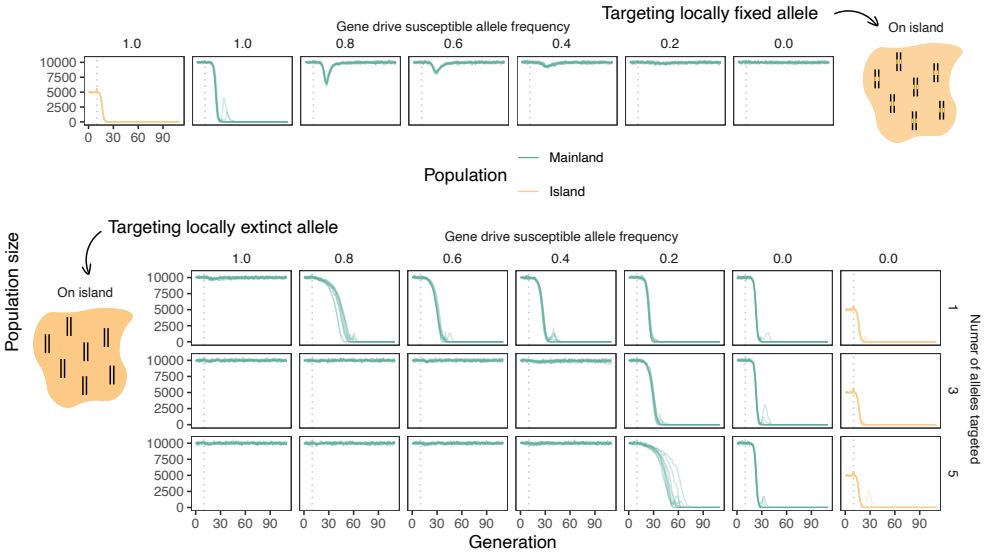


Figure 6.8. Population genetic modelling of a locally extinct self-destruct gene drive. Only population suppression is shown.

a migration rate from mainland to island. We modelled a gene drive that is highly efficient, but not perfect: a cut rate of 100%, a conversion rate of 90%, an embryo cut rate of 10%, a functional resistance rate of 0%, and an initial heterozygote introduction frequency of 10%. By targeting a locally fixed allele as is usually proposed, the mainland population was not entirely suppressed unless the locally fixed allele was fixed on the mainland too. Otherwise, there was a temporary dip in population size, followed by recovery back to carrying capacity. For the drive targeting a locally extinct allele with self-destruct, we observed that if only a single such allele is targeted, the drive retains a large amount of suppressive power even if the alternate allele frequency is as high as 0.8 on the mainland. The reason why our strategy retained suppressive power in the mainland population is that the self-destruct resistant allele migrates along with the gene drive from island to mainland at relatively high rate (0.01 per generation). Therefore, the gene drive can survive for additional generations, as it can only remove itself after random segregation away from the resistant allele. The chance of the drive encountering more resistant alleles is dependent on both the pre-existing allele frequency but also the relative influx of resistant alleles from the island.

A strategy we modelled subsequently was to simply target more locally extinct alleles, all

unlinked and thus randomly segregating. In doing so, offspring from a gene drive and wild type cross would have, for example, three or five self-destruct alleles, and the gene drive could only survive by segregating with all locally extinct alleles, the chances of which are very slim (Figure 6.8). This way, the gene drive has no considerable impact on mainland population size unless the locally extinct allele is present at low frequency on the mainland as well. These dynamics might be preferable over the original locally fixed allele strategy where the mainland population may be suppressed to a large extent, depending on the allele frequency (Figure 6.1).

However, there are also significant practical downsides to this strategy. First, whether three or five such haplolethal variant sites exist that could be targeted might be unlikely. Second, in the original strategy, there is not a real risk of complete suppression of the mainland population, whereas that is the case in this LEA strategy, especially when allele frequency differences are subtle. Third, perhaps the ultimate flaw of this design is that gene drive localisation is contingent on a certain gene drive component (the self-destruct mechanism) remaining functional for many generations. Should the drive migrate to a mainland population and the self-destruct mechanism is somehow broken (for example, a gRNA or its promoter is mutated), the gene drive will lose its localisation and allow the unlimited spread of the gene drive in the mainland population. Although this problem can perhaps largely be mitigated by separating the gRNAs across a set of self-destruct constructs, this is not an ideal scenario as resistance can form against each of these loci sequentially. For a safety mechanism, it is clearly undesirable to contend with issues of this kind and it is better to have robust and reliable strategies. Finally, having to built a gene drive with such large amounts of gRNAs (targeting three or five genes and likely having to multiplex at each to avoid the formation of resistance), will incur considerable inefficiencies; studies employing gRNA multiplexing have reported a Cas9 saturation effect, in which the presence of multiple gRNAs leads to a proportional reduction in cutting efficiency at all targeted loci (S. E. Champer et al., 2020; E. Yang et al., 2022). Particularly in mammals where drive efficiency is already limited, adding further gRNAs is likely not desirable (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021), even assuming they can be expressed in a stable manner (S. E. Champer et al., 2020). In any case, although this design may not provide the most desirable qualities and feasibility, there is substantial scope for creativity in multi-part gene drive designs to target locally fixed alleles.

6.5 Conclusion

In conclusion, the classical version of targeting locally fixed alleles is unlikely to be an efficient and widely useable strategy for gene drive safeguarding. This unlikelihood is not only due to drive design constraints and potentially unacceptable ecological consequences of escapes, but also the limited likelihood that locally fixed alleles evolve within an anthropogenic time-scale in the first place. First, we have demonstrated that we can expect large shifts in allele frequencies between island and mainland populations only in specific, and usually unrealistic, scenarios. Second, we have outlined a two-target drive design that may be localised through these shifts in allele frequency, but we primarily note associated risks and limitations.

Our island invasion model necessarily lacks some ecological realism. First, a factor that we have not addressed in our model is that sampling individuals for an invasion event is likely not a random draw from a large population (Alves et al., 2022). Spatial structure in a population could lead to biased sampling of invading individuals as, for example, a harbour population might have an allele that does not occur in the rest of the mainland, and those individuals may be more likely to invade islands as stowaways on ships. Furthermore, animal personality has also been shown to contribute to migration patterns (Nilsson et al., 2016), potentially leading to biased migration of certain genotypes. Perhaps, these factors make the formation of locally fixed alleles more likely when considering the mainland population at large (as opposed to only the source population). Second, in our model we have not considered the effect that invasive species may have on each other. Russell et al. (2011) have shown that island adaptation may be prevented when a range of invasive species invade at the same time, as they will likely interact and compete, thus preventing the evolution of naivete that is often observed on islands. This factor might reduce the likelihood that locally fixed alleles form through adaptive evolution.

An important consideration for the likelihood that locally fixed alleles can be targeted by a gene drive is that, in the ideal scenario, they should alter the protospacer adjacent motif (PAM) required for CRISPR activity, because this generates a binary cut/no-cut outcome with minimal intermediate cases. Nucleotide changes in the gRNA target sequence immediately proximal to the PAM can also effectively prevent cutting in most cases, while substitutions at the distal end are generally less reliable, as Cas9 often still cleaves despite

several mismatches (T. Zheng et al., 2017). Consequently, the allele either has to disrupt an existing PAM site or perhaps a proximal nucleotide in the gRNA target region, which is a substantial constraint. In mouse populations of Australian islands, applying PAM-based filtering step markedly reduced the number of suitable fixed alleles, underlining how restrictive this requirement is (Oh et al., 2021). This limitation might be alleviated to some extent by using alternative Cas proteins with different PAM specificities.

For gene drive safeguarding in general, temporally self-limiting gene drives such as daisy drives are perhaps more realistic and reliable strategies to limit the potential for undesirable consequences than one based on locally fixed alleles. Daisy drives separate the two essential parts of a gene drive, Cas9 and gRNAs, and drive only one of them (Guo et al., 2025; Min et al., 2017a, 2017b; Verkuijl et al., 2022). Therefore, the gene drive will lose its power to spread over time and be removed from a population. In the case of gene drive escapes, this process will likely have less impact on native populations and be more reliable than a locally fixed allele strategy. Other non-drive strategies for population control are also promising, such as genomic editors that only induce a genetic load but do not spread themselves (M. L. Johnson et al., 2024; Willis & Burt, 2025). These designs are intrinsically constrained, although they may be less desirable in terms of the trade-off between management cost and suppressive power. Further, the feasibility of such designs remains to be tested, as it is unclear whether a range of genes can be targeted at the same time while retaining viability.

For any multi-target gene drive, the limited set of genes that can potentially be targeted by gene drives could be a non-trivial challenge. Ideally, targets should be fully haplosufficient, meaning one copy is enough for a fully viable phenotype, yet whose complete loss (bi-allelic knockout) produces complete sterility or lethality (Clark et al., 2024). Crucially, this sterility or lethality should not be associated with phenotypes that compromise animal welfare, as one of the purported advantages of gene drives over conventional control methods is the potential to reduce negative welfare impacts. In multi-part gene drive strategies, compounding fitness effects may further exacerbate such issues. Indeed, unexpectedly high fitness costs have already been observed for multi-target gene drive designs in *Drosophila melanogaster*, underscoring the importance of this factor (Faber et al., 2024; Guo et al., 2025).

In general for biological invasions, many uncertainties exist. Relevant factors including the

parameters in our model are hard to estimate for real-world populations and require extensive sequencing efforts to identify. Such sequencing projects can be highly beneficial, both for gaining information with which to prevent or manage invasions, as well as to inform potential future gene drive application. As mentioned above, migration/dispersal will be one of the key parameters determining gene drive success or failure (J. Champer, Kim, et al., 2021) and suppressive power (North et al., 2020), while these effects are complex to understand and often hard to predict (Jonzén et al., 2011). Sequencing invasive populations at large scale is highly valuable to estimate these parameters and to better understand invasion genomics from a fundamental perspective, although this method is limited in its resolution. Capture-Mark-Recapture and GPS telemetry studies of invasive species should provide more detailed information (Makuya & Schradin, 2023).

The benefits and risks associated with gene drives remain a matter of active debate (Boersma et al., 2024). Rather than framing this discussion in terms of two opposing camps, we argue for a cautious, intermediate position. To date, no efficient homing gene drive has been demonstrated in mice (Grunwald et al., 2019; Pfitzner et al., 2020; Weitzel et al., 2021). It is therefore reasonable to prioritise proof-of-concept studies before focusing extensively on containment measures in most systems. Proponents of gene drives should moderate claims about imminent conservation benefits, while critics should recognise that most currently existing constructs do not have the power to bring about negative ecological impacts. The closest we are to an efficient gene drive to aid island conservation is based on a natural gene drive in mice, the *t*-haplotype (Gierus et al., 2022), but more extensive testing is necessary to determine its potential in a population setting. In any case, advancing our understanding of the ecology of invasive species is intrinsically valuable and will be essential if gene drives are to eventually be deployed in a controlled manner to support conservation efforts.

Acknowledgements

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Data and code availability

Data and code are available on GitHub (https://github.com/NickyFaber/LocallyFixedAlleles).

Author contributions

N.R.F. conceptualised the study in collaboration with A.C.C.. Modelling and analysis was carried out by N.R.F.. Writing was done by N.R.F., with feedback from A.C.C., B.A.P., B.J.Z., J.H., and J.C..

"Kya knew judgment had no place here. Evil was not in play, just life pulsing on, even at the expense of some of the participants. Biology sees right and wrong as the same color in different light. Nothing seemed too indecorous as long as the tick and the tock of life carried on. She knew this was not a dark side to Nature, just inventive ways to endure against all odds."

— Delia Owens, Where the Crawdads Sing

"We may not be able to restore the Onondaga watershed to its pre-industrial condition. The land, plants, animals, and their allies among the human people are making small steps, but ultimately it is the earth that will restore the structure and function, the ecosystem services. We might debate the authenticity of the desired reference ecosystem, but she will decide. We're not in control. What we are in control of is our relationship to the earth. Nature herself is a moving target, especially in an era of rapid climate change. Species composition may change, but relationship endures. It is the most authentic facet of the restoration. Here is where our most challenging and most rewarding work lies, in restoring a relationship of respect, responsibility, and reciprocity. And love."

— Robin Wall Kimmerer, Braiding Sweetgrass

Chapter 7

General Discussion

7.1 In this thesis

In this thesis, I have investigated the evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management. As described in the introduction, my work can be classified into three themes that fall under this broader goal: evolutionary stability, efficiency, and safety (Figure 7.1).

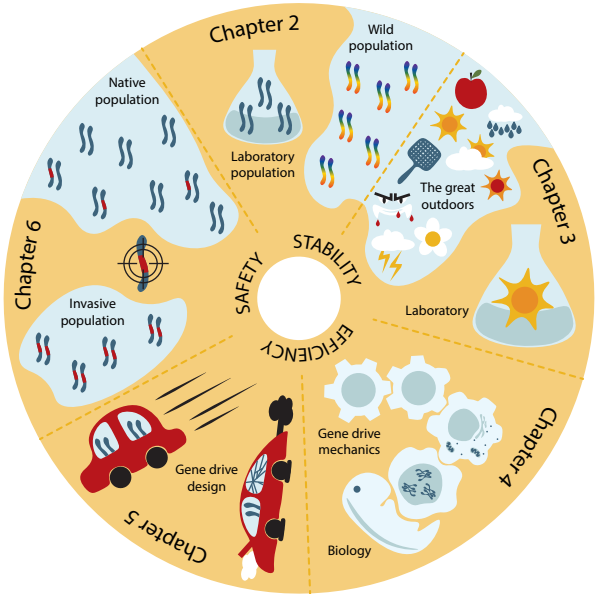


Figure 7.1. Graphical overview of this thesis.

In order for a gene drive to achieve its intended goal, whether population suppression or population modification, it should ideally remain stable over time. Much attention has already been paid to a simple form of resistance that can be generated at the drive's target site when homing activity fails. In **Chapter 2**, I have investigated a more complex, potentially polygenic type of resistance that may arise based on standing genetic variation in a population. However, I found no evidence that the standing genetic variation of several European populations of *Drosophila melanogaster* could lead to varying gene drive efficiencies. At the same time, even while using a gene drive that targets an extremely conserved gene to prevent simple resistance alleles from arising, I found several mutations in the drive's target sequence in offspring where few were thought to be viable. Therefore, I conclude that resistance is will likely still arise from gene drive activity itself, underscoring an unresolved

inefficiency in this technology.

Besides being evolutionarily stable across time, gene drives should also be stable across environments. As all gene drives are currently only assessed in homogenous laboratory environments, in **Chapter 3**, in collaboration with several BSc students, I varied a single environmental factor relevant for *D. melanogaster* in the laboratory (yeast concentration) and measured its impact on gene drive efficiency. I have found that the gene drive performed with significant variability across environments. Moreover, despite testing a modification gene drive that does not induce fitness costs on purpose, I found that the gene drive underperformed such that it could not spread through a population, while it was seemingly efficient in single crosses in terms of inheritance rate and numbers of offspring. Thus, this finding clearly indicated an unaccounted fitness cost for the gene drive carriers. This result, based only on varying the concentration of yeast in the fly medium, indicates that the fitness of drive carriers measured in the laboratory may not translate reliably to a similar fitness in the field under realistic, variable conditions. It seems that the gene drive field could be severely overestimating the fitness of their drive carriers in single crosses, although this finding may also be limited to gene drives targeting essential genes. In general, I suggest that most gene drives are likely not yet ready for open field trials and should be tested in small-scale, contained outdoor trials first (Hammond, Pollegioni, et al., 2021).

As detailed before, gene drive stability and efficiency are inherently linked; not only does the likelihood of a gene drive spreading decrease with lower efficiency, simple resistance is also more likely to evolve. Especially for suppression gene drives, it is extremely important that they are highly efficient, as they otherwise do not have the power to spread or have any suppressive power to reduce an invasive pest population in size. There are many technical reasons related to this inefficiency in certain species (Verkuijl et al., 2022), but in **Chapter 4**, in collaboration with Lynn den Otter (BSc), I theorise why gene drives may only show low or medium efficiency in certain (but not all) species by looking into their developmental biology. We find many biological factors that the gene drive field has not explicitly taken into account up until now, reflecting that most construct designs are tested primarily on a trial-and-error basis. I believe that by considering species' developmental and genome biology, much greater gene drive efficiencies can be achieved.

Should these efficiency improvements prove impossible in some species, however, in **Chapter 5**, I have theorised a gene drive design that may still effectively suppress invasive pest

populations despite this challenge. This design has two genomic targets instead of one: one to spread itself and another to induce female sterility. Based on population genetic modelling, this design has more suppressive power than standard drives that combine the two functions in one. Additionally, this gene drive is more evolutionarily robust against resistance alleles, as it allows for more flexible targeting of genomic sites via gRNA multiplexing while not reducing homing efficiency (S. E. Champer et al., 2020). In collaboration with Dr. Xuejiao Xu, we have tested the efficiency of this design in *D. melanogaster* in the laboratory, and show that unfortunately, there were unexpectedly high fitness costs associated with the drive, wherefore it was not able to spread. I hypothesize that this finding may indicate a more general limitation of multi-part gene drive designs, as fitness costs associated with each part could have compounding negative impacts.

If homing gene drives ever achieve the efficiency they were initially thought to have, they will be in need of reliable safety mechanisms to make them spatially or temporally self-limiting. In **Chapter 6**, I have demonstrated that a strategy based on locally fixed alleles is highly unlikely to be a realistic and reliable way to safeguard gene drive technology. Although there may be aspects to biological invasions that promote the genetic differentiation between native and invasive populations such as genetic bottlenecks and small population sizes in the invaded range, most invasive species have only been present on islands for an evolutionarily extremely short timespan; only a few hundred years have passed after most invasions during colonial travel. Via population genetic modelling I demonstrate that locally fixed alleles are highly unlikely to form over this time-span. The field should proceed down different avenues for localising this technology and reducing associated risks, while sequencing invasive populations remains highly valuable.

7.1.1 General conclusion

In general, I conclude that long-term evolutionary processes are unlikely to be highly relevant for gene drive technology: pre-existing genetic diversity will probably not provide a broadly useful basis for gene drive localisation mechanisms, nor will it be a major barrier in terms of efficiency through selection for resistance based on standing genetic variation. Rather, it is the mechanistic aspects of gene drive technology at smaller scales (molecular, cellular, and tissue-specific) that could benefit from further optimisation, as gene drives can suffer from low homing rates and subtle, yet impactful, fitness costs to their carriers.

Up until now, many gene drive constructs have resembled coarse instruments applied to delicate systems: insufficiently precise and prone to inducing unintended fitness costs. I believe that it is time to engage more deeply with the biology of each target species, rather than continuing to rely on optimising technical aspects via trial-and-error, to be able to design gene drives with the efficiency, stability, and safety that would make them a viable management tool for invasive pests.

7.2 What is next for gene drive technology?

Homing gene drives have come a long way over the last thirteen years since the advent of CRISPR-based genome editing, both in terms of general design and efficiency optimisation (M. A. E. Anderson et al., 2023; Du et al., 2024; Kyrou et al., 2018; Prowse et al., 2017). Nevertheless, in many species, substantial optimisation is required before gene drives may be considered for field trials, let alone practical application (Weitzel et al., 2021; X. Xu et al., 2022). To increase homing efficiency, the road ahead seems clear: namely, the gene drive's Cas9 promoter has been shown to be key in determining efficiency (M. A. E. Anderson et al., 2023; Du et al., 2024; Y. Wu et al., 2025). I believe single-cell sequencing to be the most promising strategy to find promoters that can direct gene drive activity with the necessary precision in terms of timing and level of activity (Grunwald et al., 2022; Verkuijl, 2023). Single-cell sequencing can provide detailed information on timing expression and levels at specific germline cell stages, as well as reveal undesirable expression in non-germline tissues (Page et al., 2023; Y. Wu et al., 2025).

Another limitation for efficiency is that currently, no perfect solution exists to prevent the formation of resistance alleles, although the rate at which these resistance alleles persist over generations has been greatly reduced by targeting conserved genes and using gRNA multiplexing (J. Champer, Yang, et al., 2020; S. E. Champer et al., 2020; Kyrou et al., 2018; E. Yang et al., 2022). Nonetheless, there is a growing scepticism on whether gene drives can be the large-scale pest management technique they were initially proposed to be (Esvelt et al., 2014). If efficiency limitations persist, gene drives may still be useful as supplementary tools, complementing conventional control methods by eliminating the last remaining individuals in invasive populations (Birand et al., 2025). At such reduced scales, gene drives or non–gene drive forms of genetic biocontrol do not require the same level of power to spread or stability over time, and larger releases might be acceptable once an invasive pop-

ulation is already heavily suppressed, to avoid imposing substantially more ecological pressure beyond an already unacceptable baseline.

For both homing efficiency and stability in the face of resistance, I believe that there is significant promise in increasing a drive's suppressive power by targeting more than one gene (Faber et al., 2024; Kandul et al., 2021; Willis & Burt, 2021). Furthermore, this two-target strategy can also be used in combination with natural gene drives, as has been demonstrated by using the *t*-haplotype in mice (Gierus et al., 2022), a species in which CRISPR-based homing gene drives have been largely unsuccessful thus far (Pfitzner et al., 2020; Weitzel et al., 2021). However, such two-target drives may also have double the fitness costs to contend with (Faber et al., 2024; Guo et al., 2025). For many gene drives in general, fitness costs are an ongoing challenge, the source of which remains somewhat unclear (Chapter 3; Langmüller et al., 2022), but seems at least related to the gene drive's target gene (X. Xu et al., 2024).

In theory, in the case of invasive species, there is a risk of long-range, indefinite spread beyond the invasive population (Esvelt & Gemmell, 2017). For some species, including mosquitoes, there is the additional risk of hybridisation with closely related species and the potential spread of the gene drive beyond the target species (Connolly et al., 2023). These are valid concerns and require extensive risk assessment by ecologists and geneticists, similar to classical forms of biocontrol using natural enemies (Frieß et al., 2023; Heimpel et al., 2024). At the same time, as long as the efficiency and stability of homing gene drives remain as fallible as they are at present, one consequence is that the associated ecological risks are likely to be substantially reduced. However, this propensity for inefficiency or failure is not a robust nor desirable safety mechanism, as it can also undermine the intended pest management benefits. Self-limiting and threshold-dependent drives may substantially mitigate these concerns, as they afford greater control over the spatial and temporal extent of gene drive spread and its impacts, but they still require improved efficiency and testing (Dhole et al., 2019; Metzloff et al., 2022; Min et al., 2017a, 2017b; Noble et al., 2019).

Besides gene drive–intrinsic factors, we know that from an ecological and evolutionary perspective, there are many important life history factors that will determine the success or failure of a gene drive (Wilkins et al., 2018). Theoretical modelling has indicated especially spatial migration to be such factor (Dhole et al., 2020). In spatial models, the impact of fitness costs is exacerbated, and many gene drives show considerably different dynamics in a

spatial than in a panmictic model (X. Zhang et al., 2024). The dynamics of self-limiting and threshold-dependent drives can therefore also change considerably in spatial settings (Pan & Champer, 2023). Intuitively, it makes sense that when a species migrates longer distances, the gene drive can spread faster to new areas and thus be more effective. However, there will also be more risk of wild-type individuals migrating beyond the moving front of gene drive spread. Migration in continuous space has been shown to cause the phenomenon of "chasing", where an extirpated area is continuously colonised by wild-type individuals, followed by gene drive individuals, back to extirpation, and so on (J. Champer, Kim, et al., 2021). It is unknown to what extent landscape complexities will contribute to or take away from this phenomenon, but there is increasing amounts of effort committed to modelling populations more realistically in spatial environments (S. E. Champer, 2025).

Additional layers of complexity arise when we account for the broader ecological context in which a gene drive is released. A significant complicating matter is the presence of multiple invasive species in one area. For example, Rakiura is an island in New Zealand that is invaded by cats, rats, possums, and hedgehogs, whose eradication is in the planning phases (Bell & Bramley, 2013). However, there are major unanswered questions on what the best strategy would be: could removing cats cause an explosion in the number of rats? Could removing cats and rats finally allow mice to invade and settle on the island as well? Should all these species be removed at the same time? Much research is needed on the relationships these invasive populations have with each other. For gene drives, a theoretical modelling study has shown that species competing with each other can make a gene drive more successful, even at lower efficiencies (Y. Liu et al., 2023), but much further work is needed.

Ultimately, the goal is not simply to reduce pest populations, but more precisely, to reduce the harm they cause. For example, even an inefficient gene drive that immunises mosquitoes against malaria may, despite failing to reach fixation, still be able to eliminate the malaria parasite from a given area (Allegretti et al., 2025). Gene drives may also act synergistically with conventional control methods to reduce malaria transmission (Abraham et al., 2025; Hancock et al., 2024). In all cases, it is important that the gene drive field maintains a clear focus on these intended public-health or conservation outcomes, regardless of whether gene drives play a leading or supporting role.

Finally, a major area for future work is on the social side of gene drive research, includ-

ing public engagement and governance. Studies have been conducted to scope the public perception in various, specific contexts (MacDonald et al., 2020; Schairer et al., 2022; Sogodogo et al., 2025), including indigenous people's perspectives (Palmer et al., 2020). In some cases, people's reaction to gene drive technology was related to their broad worldviews and values related to nature, but in other cases, people's concerns were related to pragmatic aspects such as cost and efficiency. For the Māori people in New Zealand, including indigenous autonomy and customs in the governance of gene drive technology were paramount.

In general, it remains unclear how gene drive scientists should go about the communication of their work, given the complexity of constructs and population genetics (Nerlich & Stelmach, 2022; Schairer et al., 2020). Furthermore, it is unclear from an ethical standpoint how broadly people's opinions should be solicited: local stakeholders only, region- or country-level, or even broader? (Boersma et al., 2024). This latter factor is especially important in the context of initial field trials, as such trials may result in unpredictable outcomes, from higher-than-expected efficiency to complete gene drive failure, leaving behind traces of genetic modifications in wild populations. Using island communities as "experimental playgrounds" is especially undesirable (Boersma et al., 2024). Fair partnership and transparency between all parties involved is essential to the process of eventually testing and deploying gene drives in the field (Kormos et al., 2022; Long et al., 2020). Among several factors related to safety, it is important to consider the credibility of existing regulatory structures in a country (Connolly et al., 2024), a fact that was put starkly into focus this year when a planned gene drive field trial with malaria mosquitoes came to an abrupt end in Burkina Faso (Kupferschmidt, 2025). All in all, there is much work to be done on the regulatory aspects of gene drive technology, whether for field trials or for gene drive deployment.

7.3 A gene drive related perspective on invasive species

In conservation and ecological restoration, three classes of species are widely discussed: umbrella species, keystone species, and flagship species (Kau et al., 2025). Umbrella species are species with a large and broad range, whose conservation will automatically lead to the conservation of many other species (for example, panthers and snow leopards). Keystone species are species that play a disproportionally large role in ecosystem functioning (the

most famous example being wolves in Yellowstone National Park, but including also other ecosystem engineers such as beavers). Flagship species are species that are unusually charismatic, which may make people (disproportionally?) enthusiastic about conservation in general (examples include the kākāpō and the giant panda, the latter also being an umbrella species).

With the advent of gene drive technologies, perhaps we need a term and set of defining criteria in the general framework of conservation for invasive species that have such a far-reaching negative impact that an entire species or species group is threatened with extinction. Per definition, all invasive species cause negative effects, but inevitably, some will have more extensive detrimental impacts than others. A similar term primarily used in invasive plant research is used to indicate invasive species that transform entire ecosystems: transformer species (Richardson et al., 2000). These species cause major shifts in ecosystem functioning (for example, fire regimes, nutrient cycles, or water retention). On many islands, native flora and fauna have already been decimated and may go extinct unless highly intensive management of some invasive species continues (Fernández-Palacios et al., 2021). To this end, I would like to propose the term "gravestone species" as the negative counterpart to the disproportionally positive impacts that keystone species have. These gravestone species would probably include invasive species such as rats, feral cats, and wild pigs (Luque et al., 2014). The International Union for Conservation of Nature (IUCN) is best positioned to take a leading role in such classification efforts, but members are currently deeply divided on the permissibility of considering synthetic biology as conservation tool (International Union for Conservation of Nature (IUCN), 2025a, 2025b).

I believe we should focus on ecology first, and only then look at what tools might help to achieve our conservation goals. To this end, a major benefit that the introduction of a term such as gravestone species might have is that we can collect an objective set of requirements that will (or will not) qualify certain invasive species for gravestone species status. This process may help the assessment of whether or not technologies such as gene drives are appropriate and proportional for consideration, as they can arguably be drastic measures from an ethical point of view (Boersma, 2022) and, more importantly, come with ecological risks (Frieß et al., 2023). For example, the grey squirrel is an invasive species in the United Kingdom and it negatively impacts the native red squirrel (through direct competition and the spread of disease), forest conservation and restoration (through bark strip-

ping), and human property (Mountford et al., 1999; Tompkins et al., 2003; Williams et al., 2010). However, whether the grey squirrel would qualify as a gravestone species is debatable for many reasons. First, there are known ways of sustainably controlling or eradicating grey squirrel populations. Recently, grey squirrel numbers declined dramatically while red squirrels simultaneously re-colonised major areas of Ireland due to the reintroduction of a native predator: the pine marten (Sheehy & Lawton, 2014). Second, red squirrels suffer significantly from impacts besides the grey squirrel's presence such as habitat changes by the forestry industry, so altering tree composition may also offer sustainable conservation outcomes (Shuttleworth et al., 2012). Furthermore, there has never been sustained motivation from the UK government to control grey squirrels (Sheail, 1999) and in fact, up until 50 years ago, red squirrels were persecuted by farmers with governmental support due to property damage (Holmes, 2015). Importantly, public support for grey squirrel control is also low (Dunn et al., 2018). So, to gene drive or not to gene drive? These are complex considerations that ecologists are best positioned to provide guidance on rather than gene drive researchers.

For some impactful insect crop pests, a case for a gravestone species might be made indirectly. If an invasive species such as *Drosophila suzukii* were to block the transition from a system based on chemical pest control to a more biologically based one (Nederlandse Voedsel- en Warenautoriteit (NVWA), 2024), thereby increasing the risk of extinction for some endangered insects, then genetic biocontrol or even gene drives could become valuable methods of pest management (Buchman et al., 2018; Yadav et al., 2023). However, such arguments could also be advanced primarily in service of financial interests within agricultural systems. Many invasive species are, moreover, opportunistic beneficiaries of human-driven environmental modifications such as monocultures and could otherwise not be so prolific. As the consequences of current agricultural practices become increasingly apparent, it is important that gene drive technology does not hinder a transition to more nature-inclusive and sustainable forms of agriculture.

The reason that this gravestone species classification might become valuable is related to a criticism that can be levelled at the gene drive field: it can potentially behave as a solution in search of problems, rather than the other way around. Although gene drives were already in development for malaria mosquitoes before the advent of CRISPR in 2013 (Burt, 2003; Deredec et al., 2008; Windbichler et al., 2011), this development has ramped up consid-

erably in a wide range of species since, largely because of its newfound cost-effectiveness. Now, any species that is a crop pest, whether native or exotic, or any exotic species, whether of major or minor impact, might have a case made for the development of a gene drive against it. Indeed, studies have found that only a narrow group of scientists is involved in the debate and narration of gene drive technologies (de Graeff et al., 2019; Rabitz, 2024). More importantly, with several patent holders and start-up companies operating in the absence of comprehensive regulatory frameworks, this constitutes a non-trivial risk (de Graeff et al., 2018). Scientists in the gene drive field may be particularly at risk of misrepresenting invasive species problems, as they are often required to begin their grant applications and papers with descriptions of how important and unmanageable a problem is that they aim to address. In light of these risks, gene drive researchers and companies should not be the primary leaders in public engagement or policy making, but rather act as consultants with measured input. Instead, problem definition should come first, with ecologists playing a central role.

One widely raised argument against gene drive usability is that many regions have limited control over the continued introduction or migration of invasive species (Boersma et al., 2024). Obtaining robust migration data can be exceedingly difficult, as it typically requires labour-intensive capture–mark–recapture studies, GPS telemetry studies (also effective for small rodents, Makuya and Schradin, 2023), or low-resolution estimates based on genetic divergence between regions. Suppose a gene drive is implemented, with all the necessary development, optimisation, field testing, public engagement and legal preparations completed, and it successfully eradicates an invasive population over a multi-year timespan; it would represent a major loss of effort if the species were to simply migrate back into the area and begin colonising it anew, similar to what has occurred with screw worm flies following intensive sterile insect technique (SIT) programmes (Zaldivar-Gomez et al., 2025). Gene drives are likely not suitable as continuous management solution, given their susceptibility to resistance allele formation and associated biosafety risks. Gene drive constructs can, in principle, harmlessly co-evolve through redesign of gRNAs or targeted genes (in contrast to chemical pesticides), but extended use may also bring extended risk to other populations. Measures that reduce propagule pressure, that is, the number of (continuously) invading individuals whether via autonomous or human-mediated migration, such as reducing high-risk pathways in global trade, are likely to be among the most effective ways to address invasive species problems at the source and should be prioritised before resorting

to eradication efforts as intensive as gene drive deployment (Lockwood et al., 2005).

7.4 In defence of trying

Gene drives are, ten years after the field has rapidly expanded, still an unproven and uncertain technology. They do not work in the simple, clean sense that one might wish for: there are many inefficiencies, untested contingencies, unknowns, and many possible routes to failure. Some biologists I have spoken with argue that gene drives will likely never be reliable tools for conservation by controlling invasive species, because evolution will always find a way around them. There is, at best, a great deal we do not yet know.

At the same time, there is a clear idea of what "good" gene drives could do. In principle, they might allow us to move away from broad-spectrum pesticides with many harmful off-target effects, and move us towards more targeted and species-specific interventions. For some conservation cases that currently look somewhat hopeless, including emblematic species such as the kākāpō, unable to co-exist with introduced predators, gene drives can represent a fragile kind of hope.

The risks of this technology are equally real. For many people, gene drives are a symbol of a new frontier: the genome as the next area for ever-expanding human control (Callaway, 2016; Cobb, 2022). We risk irreversibly altering the genetic make-up of a wild population. A series of failed or escaped gene drives, assuming they will be self-limited, could result in a host of broken construct pieces drifting around in populations. There is already extensive evidence of resistance emerging in the laboratory; there is no reason to expect nature to be more forgiving outside in the field. More broadly, the histories of both chemical and biological pest control contain many examples in which interventions produced unexpected and sometimes disastrous consequences (Shine et al., 2020). It is understandable, and reasonable, that people view yet another technological fix with suspicion.

Critics of techno-fixes remind us that systemic problems are rarely resolved, if not sustained, by technical solutions alone. Invasive species are tightly entangled with complex colonial histories and global trade (Skotnes-Brown & Lynteris, 2025), and our biodiversity crisis is also linked with current-day agricultural practices (Díaz & Malhi, 2022). The rate at which invasive species are introduced around the world is increasing (Seebens et al., 2020), and in that sense one can argue that focusing on a genetic tool for one species addresses

symptoms rather than causes. Yet it is also true that our ecological understanding, regulatory frameworks, and ethical awareness are not the same as they were at the time of earlier introductions. In recent decades, biocontrol that introduces natural enemies into foreign habitats to control invasive species has, in many cases, been highly effective (Barratt et al., 2018). The context in which we consider new interventions has changed (Boersma, 2022), even if not as quickly or as deeply as one might wish.

Personally, I find it difficult to accept the damage we have already done and let some species disappear "with love". I struggle with the idea that their future may only be as remnants in zoos or tightly managed reserves, but no longer as truly wild animals. I have focused my education on genetics and gene drives; this is the place from which I have chosen to push against the tide of our ongoing biodiversity crisis, potentially in vain if gene drives never prove efficient enough or, even if so, are not used after all. Working on such a technology does not excuse us from confronting structural injustice, unequal vulnerability, or the political economies that produced (and in many cases, continue to produce) ecological damage in the first place. It is one kind of response among many (Leclère et al., 2020): an attempt to reduce specific impacts in specific contexts, under conditions of uncertainty.

There is, I think, a kind of beauty in the way many people continue to try with persistent effort to save conservation cases that may appear almost hopeless. We know what we have done and continue to do as the climate changes and ecosystems are transformed in ways that feel overwhelming (Leclère et al., 2020). Humans contain multitudes this way: capable of care and cruelty, curiosity and denial, creativity and conformism. To work on gene drives for conservation is to inhabit that tension very directly.

I also feel a similar tension in my own life. The ideal of treading lightly on the earth, walking more, consuming less, sits uneasily alongside an academic career that demands mobility, productivity, and constant indoor work. Conferences, courses, and collaborations pull people across countries and continents, with all the environmental and personal costs this entails. The dissonance between the world I would like to help protect and the practices that academic life currently normalises is sharp, and not easily resolved. Such personal dilemmas mirror broader considerations in conservation: even when technologies such as gene drives offer potential benefits, they are embedded within systems that are imperfect and morally complex.

I believe that some of the damage humans have done can be mitigated, and that fixing our relationship with nature is possible, even if it is not guaranteed. Gene drive technology cannot "save" us from our biodiversity crisis, and it may well prove unsuitable or unjustifiable in many cases. But the effort to explore them critically, transparently, and with attention to systemic problems is, for me, a meaningful form of trying to right a small part of a much larger wrong.

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"De aarde zal weer bruisen, als we stoppen de kiem te smoren die niet buigt. Want alles hangt met alles samen en we buigen heel ver, tot we barsten. We vormen vóór we begrijpen. We zien vóór we kijken. We raken aan en we wijken, want alles hangt met alles samen en iedereen groeit naar het licht."

— Mariken Heitman, Wormmaan

Translation in English by Niek Miedema

"The earth will once again effervesce, if we stop smothering the seedling that will not bend. For everything is connected to everything else, and we bend very low, until we break. We form before we understand. We see before we look. We touch and we recede, for everything is connected to everything else, and everyone grows towards the light."

Summary

Summary

Gene drives are engineered genetic elements that can spread a trait quickly through a population, even when they are designed for population control by incurring a fitness cost. Thus, they could improve human health and conservation through the sustainable control of invasive pest species. Although gene drives are widely studied in terms of inherent efficiency and biotechnological feasibility, few studies have focussed on the evolutionary aspects of this technology. Currently, most studies test gene drives in small cage populations using genetically homogenous lab strains. There are many open questions about the efficiency of gene drives in genetically diverse populations, the evolutionary stability over long periods of time, and the practical applicability of safety mechanisms based on the evolutionary genetics of invasive populations.

In this thesis, I have investigated the evolutionary prerequisites for safe and effective use of gene drive technology in invasive pest management. My work can be classified into three themes that fall under this broader goal: evolutionary stability, efficiency, and safety.

For gene drives to achieve population suppression or modification, they must remain evolutionarily stable. In **Chapter 2**, I tested whether standing genetic variation in several European *Drosophila melanogaster* populations could affect gene drive efficiency, but found no evidence for such polygenic resistance. However, even with a drive targeting a highly conserved gene, mutations in the gene drive target site still arose, potentially indicating simple resistance. This result suggests that resistance forming from drive activity itself is the most significant evolutionary instability for this technology.

Because gene drives also need to be robust to environmental variation, in **Chapter 3** I examined how a single ecological factor, yeast availability, affects drive performance in *D. melanogaster*. Even this single change in environmental conditions caused considerable variability in drive efficiency. Furthermore, although the gene drive appeared effective in single crosses, it failed to spread in most cage experiments, implying hidden fitness costs. These results indicate that laboratory fitness estimates are likely overly optimistic, and that gene drives are not yet ready for open field trials without intermediate, contained, small-scale testing.

Achieving the necessary gene drive efficiency to enable spread through a population is still

a major challenge in many species. In **Chapter 4**, I explored how species-specific developmental biology may explain why many gene drives achieve only low or moderate efficiency. We found numerous biological factors that have been overlooked in design strategies, which are often based on trial-and-error. Taking developmental and genomic context into account during gene drive design is likely the most promising strategy to improve gene drive efficiency going forward.

When such improvements are not feasible, alternative gene drive designs may help. In **Chapter 5**, I developed and modelled a two-part gene drive that separates the spreading and sterilising functions of a gene drive for improved suppressive power. This design shows stronger suppressive potential under intermediate gene drive inheritance rates as well as a greater robustness to resistance. However, laboratory testing in *D. melanogaster* revealed unexpectedly high fitness costs that prevented spread, potentially indicating a limitation of multi-part gene drive designs.

Finally, in **Chapter 6** I assessed the feasibility of a safety mechanism that relies on locally fixed alleles. Population genetic modelling showed that such alleles are highly unlikely to form within the short time frames typical of invasive species on islands, making this strategy unlikely to be a realistic mechanism for spatial containment.

In general, I conclude that long-term evolutionary processes are unlikely to be highly relevant for gene drive technology: pre-existing genetic diversity is unlikely to provide a broadly useful basis for gene drive localisation mechanisms, nor will it be a major barrier in terms of efficiency through selection for resistance based on standing genetic variation. Rather, it is the mechanistic aspects of gene drive technology at smaller scales (molecular, cellular, and tissue-specific) that could benefit from further optimisation, as gene drives can suffer from low homing rates and subtle, yet impactful, fitness costs to their carriers. I believe that it is time to engage more deeply with the biology of each target species, rather than continuing to rely on optimising technical aspects via trial-and-error, to be able to design gene drives with the efficiency, stability, and safety that would make them a viable management tool for invasive pests. Equally important is the development of rigorous ecological risk assessments, robust governance structures, and transparent science communication to ensure that any potential deployment proceeds responsibly and with public trust.



""Gifty, how are the experiments going?" he always asks with a little wink. He seems to be one of those people who hears "scientist" and thinks "sci-fi," and his winks are to assure me that he won't tell anyone that I've been trying to figure out how to clone an alien. He and Han get along.

I wish I were trying to figure out how to clone an alien, but my work pursuits are much more modest: neurons and proteins and mammals. I'm no longer interested in other worlds or spiritual planes. I've seen enough in a mouse to understand transcendence, holiness, redemption. In people, I've seen even more."

— Yaa Gyasi, Transcendent Kingdom

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About the author

Nicky Renée Faber was born in Leiderdorp on the 27th of June, 1994. She grew up in Leiden, but moved to Wageningen in 2013 for her BSc in Biology at Wageningen University & Research. She remained there for an MSc in Bioinformatics starting in 2017, with a brief period abroad for an internship at the Roslin Institute in Edinburgh, Scotland. During this internship, she first worked on gene drives, from which the rest of her career has snowballed. Specifically, she assessed their potential to control grey squirrels in the United Kingdom using population genetic modelling. She then worked at the Roslin Institute for a short period in 2020 as a researcher, modelling gene drives in Varroa mite populations and Asian hornet populations. At the end of 2020, she started her PhD, again in Wageningen, on evolutionary aspects of gene drive technology, with an interlude in 2022 to hike the Pacific Crest Trail in the United States of America and another in 2026 to hike the Te Araroa trail in New Zealand.



List of publications

*Equal contribution

Faber, N. R., Ashok, K., Venkatesan, T., Wertheim, B., & Bulgarella, M. (2025). Leveraging advances in RNAi and CRISPR for improved biological pest control. *Current Opinion in Insect Science*, 101453. https://doi.org/https://doi.org/10.1016/j.cois.2025.101453

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Preprints

den Otter, L. R., Pannebakker, B. A., van den Heuvel, J., Zwaan, B. J., Champer, J., & Faber, N. R. (2025). Why don't mice drive? A developmental biology perspective on barriers to homing gene drive efficiency. *SSRN*. https://doi.org/10.2139/ssrn.5702523

Zhou, R.*, Du, J.*, Faber, N. R., & Champer, J. (2025). Variants in Cas9 and *nanos* regulatory elements modulate activity and reduce resistance allele formation in homing gene drive. *bioRxiv*. https://doi.org/10.1101/2025.11.04.686506

Faber, N. R., Champer, J., Pannebakker, B. A., Zwaan, B. J., & van den Heuvel, J. (2025). Investigating multiple types of resistance against a homing gene drive in European populations of *Drosophila melanogaster*. *bioRxiv*. https://doi.org/10.1101/2025.06.06.658298

Overview of completed training activities

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 30 ECTS (= 20 weeks of activities).



Writing of project proposal (6.0 ECTS)

- PhD project proposal entitled "Evolutionary prerequisites for safe and effective use of gene drives in invasive pest management."

Postgraduate courses (9.5 ECTS)

- Advanced Molecular Ecology (2021)
- Design of Experiments (2021)
- Population Genetic Modelling Using SLiM Software (2024)
- Principles of Ecological and Evolutionary Genomics (2025)

Invited review of journal manuscripts (3.0 ECTS)

- *PLOS Computational Biology*, gene drive population genetic modelling (2021)
- *Neobiota*, gene drive population genetic modelling (2022)
- *Evolutionary Applications*, gene drive population genetic modelling (2024)

Competence, skills and career-oriented activities (3.1 ECTS)

- MINDLAB (2022)
- Start to Supervise BSc & MSc Thesis Students (2023)
- Active Bystander Training (2023)
- Humour Unites: Comedy & Conversations on Diversity (2024)
- Writing Propositions for your PhD (2024)
- Critical Thinking and Argumentation (2025)
- Writing Grant Proposals (2025)

- Reviewing a Scientific Manuscript (2025)
- Last Stretch of the PhD Programme (2025)

Scientific integrity/ethics in science activities (1.4 ECTS)

- Symposium "Ethics of gene drive technologies in the Anthropocene" (2024)
- Ethics in Plant and Environmental Sciences (2025)
- Ethics and Animal Sciences (2025)

PE&RC annual meetings, seminars, and PE&RC weekends (2.7 ECTS)

- PE&RC First Year's Day (2021)
- PE&RC Day (2021)
- PE&RC First Year's Weekend (2021)
- PhD Carousel (2021)
- PE&RC Last Year's Weekend (2025)

National/local scientific meetings, seminars, and discussion groups (15.7 ECTS)

- Evolution Journal Club, Laboratory of Genetics (2021-2025)
- Dutch Entomology Day (2020-2024)
- NLSEB Annual Meeting & PhD/Postdoc Day (2023-2025)
- NLSEB Lab Rotation (2022-2025)
- NAEM (2021)
- Rewilding Symposium by Liesbeth Bakker (2020)
- 'Wild' ideas: Dialogues on Human-wildlife Interactions in a Changing World (2021)
- GeneConvene Global Collaborative Webinars - Genetic Biocontrol (2021)
- GeneConvene Global Collaborative Webinars - Ecological Modeling in Risk Assessment of Gene Drives (2021)
- GeneConvene Global Collaborative Webinars - Ecological Relationships of Mosquito Disease Vectors: Anticipating Risk Assessment of Gene Drive Technologies (2021)
- AGSX Virtual Symposium - Application of New Genomic Tools and Techniques in Arthropods (2021)

International symposia, workshops and conferences (15.6 ECTS)

- Interdisciplinary Workshop on Synthetic Gene Drives, online (2021)
- Genomic Biodiversity Knowledge for Resilient Ecosystems (G-BiKE) Webinar, online (2023)
- Society for Molecular Biology and Evolution (SMBE) Annual Conference, Ferrara, Italy (2023)
- European Drosophila Population Genomics Consortium (DrosEU) Annual Meeting, Wageningen, The Netherlands (2023)
- Gordon Research Seminar (GRS) & Gordon Research Conference (GRC) Genetic Biocontrol, Barcelona, Spain (2024)
- International Congress of Entomology (ICE), Kyoto, Japan (2024)
- European Drosophila Population Genomics Consortium (DrosEU) Annual Meeting, Lisbon, Portugal (2025)

Societally relevant exposure (0.4 ECTS)

- Newspaper article interview “Een eekhoorn van Troje” (Trouw, 2021)
- Newspaper article interview “Roeit het plaagdier straks zichzelf uit?” (Financieel dagblad, 2021)
- Magazine article interview “Steriele eekhoorn kan biodiversiteit herstellen” (Resource, 2021)
- Podcast interview "Controlling *Varroa* through genetic technology" (Two Bees in a Podcast, UF/IFAS Honey Bee Lab, 2023)

Committee work (2 ECTS)

- NLSEB Junior Board (General Member, 2022-2023)
- NLSEB Junior Board (Chair, 2023-2024)

Lecturing / supervision of practicals / tutorials (1.4 ECTS)

- Lecture in Data-Driven Animal and Plant Breeding and Genetics (University of Edinburgh, 2021)
- Lecture in Animal Genomics (University of Ljubljana, 2021)
- Lecture in Genetic Analysis, Tools, and Concepts (Wageningen University & Research, 2021)

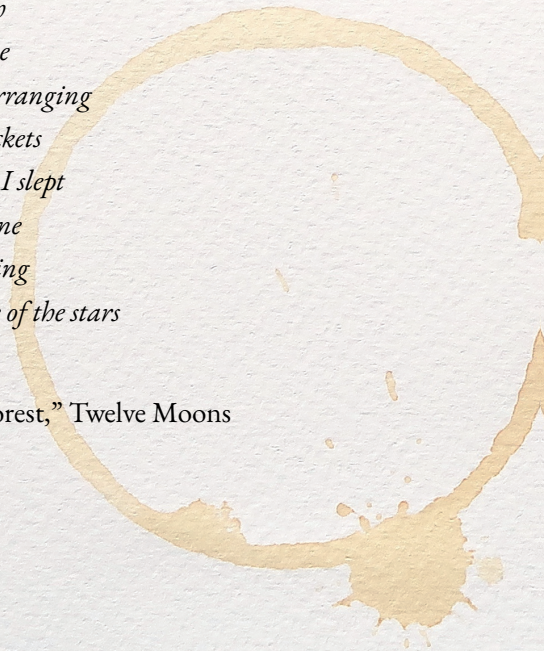
- Keystone Project Stakeholder Interviewee (University of Amsterdam, 2023-2024)
- Lecture in Population and Quantitative Genetics (Wageningen University & Research, 2025)

BSc/MSc thesis supervision (6 ECTS)

- Research topic 1: "Patent licensing strategies as a governance and regulatory tool to promote biosafety, biosecurity and ethical use of gene drive technology"
- Research topic 2: "Modelling the evolution of locally fixed alleles after island invasions for gene drive localisation"
- Research topic 3: "Effect of stress and ageing on gene drive efficiency"
- Research topic 4: "Untangling gene drive activity on a single-cell level in the *Drosophila melanogaster* germline"
- Research topic 5: "Does diet influence the spread of a gene drive through a population of *Drosophila melanogaster*?"
- Research topic 6: "Why don't mice drive? Investigating germline development of fruit flies, mosquitoes, and mice to improve homing gene drive efficiency"
- Research topic 7: "The effect of diet on gene drive efficiency and fitness in *Drosophila melanogaster*"
- Research topic 8: "The influence of ageing on the efficiency of a homing gene drive in *Drosophila melanogaster*"
- Research topic 9: "Influence of diet and introduction frequency on gene drive effectiveness in populations of *Drosophila melanogaster*"

"I thought the earth
remembered me, she
took me back so tenderly, arranging
her dark skirts, her pockets
full of lichens and seeds. I slept
as never before, a stone
on the riverbed, nothing
between me and the white fire of the stars
but my thoughts"

— Mary Oliver, "Sleeping in the Forest," Twelve Moons



The research presented in this thesis was performed at the Laboratory of Genetics at Wageningen University & Research (WUR) and was financially supported by the Graduate School for Production Ecology & Resource Conservation (PE&RC) in the Netherlands.

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