Double drives and private alleles for localised population genetic control

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Short title:

Double drives for localised population genetic control

Abstract

Synthetic gene drive constructs could, in principle, provide the basis for highly efficient interventions to control disease vectors and other pest species. This efficiency derives in part from leveraging natural processes of dispersal and gene flow to spread the construct and its impacts from one population to another. However, sometimes (for example, with invasive species) only specific populations are in need of control, and impacts on non-target populations would be undesirable. Many gene drive designs use nucleases that recognise and cleave specific genomic sequences, and one way to restrict their spread would be to exploit sequence differences between target and non-target populations. In this paper we propose and model a series of low threshold double drive designs for population suppression, each consisting of two constructs, one imposing a reproductive load on the population and the other inserted into a differentiated locus and controlling the drive of the first. Simple deterministic, discrete-generation computer simulations are used to assess the alternative designs. We find that the simplest double drive designs are significantly more robust to pre-existing cleavage resistance at the differentiated locus than single drive designs, and that more complex designs incorporating sex ratio distortion can be more efficient still, even allowing for successful control when the differentiated locus is neutral and there is up to 50% pre-existing resistance in the target population. Similar designs can also be used for population replacement, with similar benefits. A population genomic analysis of PAM sites in island and mainland populations of the malaria mosquito *Anopheles gambiae* indicates that the differentiation needed for our methods to work can exist in nature. Double drives should be considered when efficient but localised population genetic control is needed and there is some genetic differentiation between target and non-target populations.

Author summary

Some disease vectors, invasive species, and other pests cannot be satisfactorily controlled with existing interventions, and new methods are required. Synthetic gene drive systems that are able to spread though populations because they are inherited at a greater-than-Mendelian rate have the potential to form the basis for new, highly efficient pest control measures. The most efficient such strategies use natural gene flow to spread a construct throughout a species' range, but if control is only desired in a particular location then these approaches may not be appropriate. As some of the most promising gene drive designs use nucleases to target specific DNA sequences, it ought to be possible to exploit sequence differences between target and non-target populations to restrict the spread and impact of a gene drive. In this paper we propose using two-construct "double drive" designs that exploit pre-existing sequence differences between target and non-target populations. Our approaches maintain the efficiencies associated with only small release rates being needed and can work if the differentiated locus is selectively neutral and if the differentiation is far from complete, and therefore expand the range of options to be considered in developing genetic approaches to control pest species.

Introduction

Gene drive is a natural phenomenon in which some genes are able to increase in frequency and spread through populations by contriving to be inherited at a greater-than-Mendelian rate [1, 2]. Strong drive can cause genes to increase rapidly in frequency even if they also harm the organisms carrying them, and there is currently much effort trying to develop synthetic gene drive constructs (or gene drives) to control disease-transmitting mosquitoes and other pest populations that have thus far been difficult or impossible to manage satisfactorily [3-6]. If a species is harmful and subject to control measures wherever it exists, then, in principle (i.e., in the computer), highly efficient gene drive strategies can be devised that exploit natural processes of dispersal and gene flow such that relatively small inoculative releases in a few locations can lead to substantial and widespread impacts over

subsequent generations [7-9]. However, some species are pests only in a part of their range (e.g., invasive species), and other approaches are needed.

Two broad approaches have been proposed for restricting the impact of genetic control interventions to a target population. First, one can use a strategy requiring relatively large releases, which can be restricted to the target population, with any introductions into the non-target population (by dispersal, or by accidental or unauthorised releases) being too small to have a significant impact. Potentially suitable genetic constructs include those that do not drive (e.g., dominant lethals, autosomal X-shredders, or Y-linked editors; [10-12]), or those that do, but only if they are above some threshold frequency (e.g., many approaches based on the logic of toxins and antidotes; [13-15]). Some of these approaches are more efficient than others [10, 16, 17], but, by necessity, all of them require a non-trivial production and release effort.

Alternatively, one could exploit sequence differences between target and non-target populations, in which case it may be possible to retain the small release rates and overall efficiency of low threshold gene drive approaches [18, 19]. Sudweeks et al. [19] present useful modelling of this approach, considering the case where there is a locally fixed allele of an essential gene in the target population, while non-target populations carry a resistant allele at some frequency. A nuclease-based gene drive that uses the homing reaction (i.e., sequence-specific cleavage followed by homologous repair [3, 20]) to disrupt the locally fixed allele could be released into and eliminate the target population, but have little impact, or only a transient impact, on non-target populations. However, as emphasised by the authors, if the target population has even a small frequency of the resistant allele, then that allele could be rapidly selected for and the intervention fail.

In this paper we explore alternative two locus “double drive” low threshold strategies to restrict population control based on pre-existing sequence differences between target and non-target populations. All our designs are based on a division of labour between the two constructs, with one imposing a reproductive load by disrupting a gene needed for survival or reproduction, and therefore responsible for the desired impact (population suppression), and

the other responsible for the population restriction. These designs are substantially less susceptible to pre-existing resistance in the target population at the differentiated locus than single drive designs, and can even work if the differentiated locus is selectively neutral. Double drives may also be useful for population replacement. Finally, analyses of published genome sequences from island and mainland populations of the malaria mosquito *Anopheles gambiae* indicates that the sort of population differentiation we model can exist in nature.

Results

Simple double drives for population suppression

The simplest double drive designs we consider consist of one construct (call it α) inserted into and disrupting a haplo-sufficient female-essential gene, such that homozygous females die without reproducing while heterozygous females and all males are unaffected, and a second construct (β) inserted into a sequence that is significantly more common in the target than the non-target population(s). Both constructs are able to drive by the homing reaction but α can home only in the presence of β, while β may either home autonomously or rely on the presence of α. With CRISPR-based designs, α would encode its cognate gRNA, β would encode the Cas9, and either construct could encode the gRNA for the second locus (Fig 1, Designs 1 and 2). We assume the α construct has been designed such that functional resistance is not possible, though non-functional resistant alleles can arise by end-joining repair [21]. For the β construct we initially suppose its insertion site (i.e., the differentiated locus) is selectively neutral and unlinked with the α insertion site, and that differentiation is nearly complete, with the target sequence present at a frequency of 99% in the target population and absent in the non-target population (i.e., it is a virtually fixed private allele).

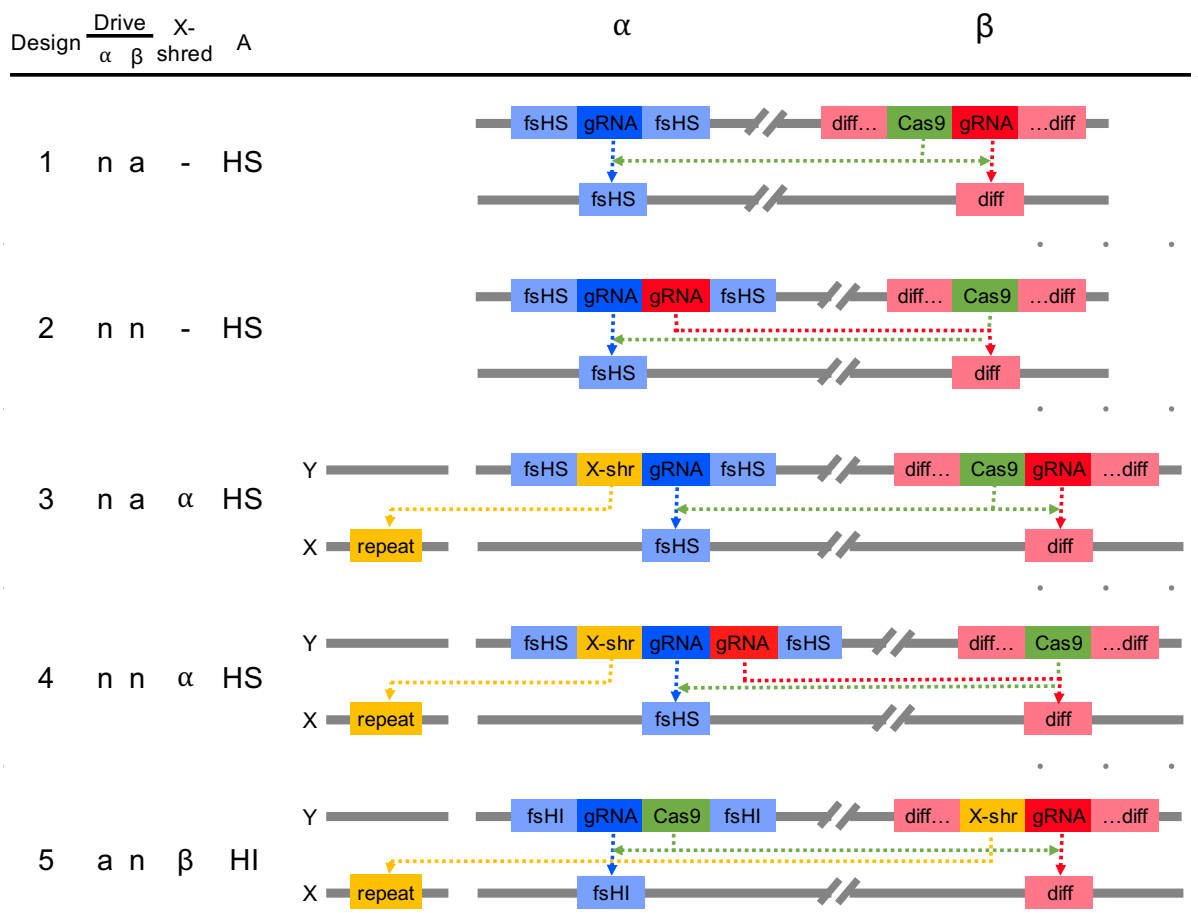


Fig 1. Alternative double drive designs for population suppression. Constructs α and β can drive autonomously (a) or non-autonomously (n); one or the other may encode an X-shredder; and the A target locus can be a gene that is haplo-sufficient (HS) or haplo-insufficient (HI) for female viability or fertility. fsHS - female-specific haplo-sufficient locus; fsHI - female-specific haplo-insufficient locus; diff - differentiated sequence; X-shr - X-shredder targeting an X-linked repeat.

Under these conditions, a small (0.1%) release of males carrying Design 1 constructs into the target population leads to both constructs rapidly increasing in frequency and, as a result, the population size crashes to a minimum size of 3.58e-6 (relative to the pre-release equilibrium) in 25 generations (Fig 2A). Depending on the initial population size and the biology of the species (e.g., whether there are Allee effects [22]), this decline could be enough to eliminate the population. However, in our simple deterministic model population elimination is not possible. Instead, the population recovers due to the evolution of resistance at the differentiated locus, leading to loss of the β construct, which then leads to loss of α, allowing the wild-type allele and population fertility to recover. By contrast, the same releases into the

non-target population have minimal effect: β cannot increase in frequency, both construct frequencies remain low, and population size is little affected (Fig 2B).

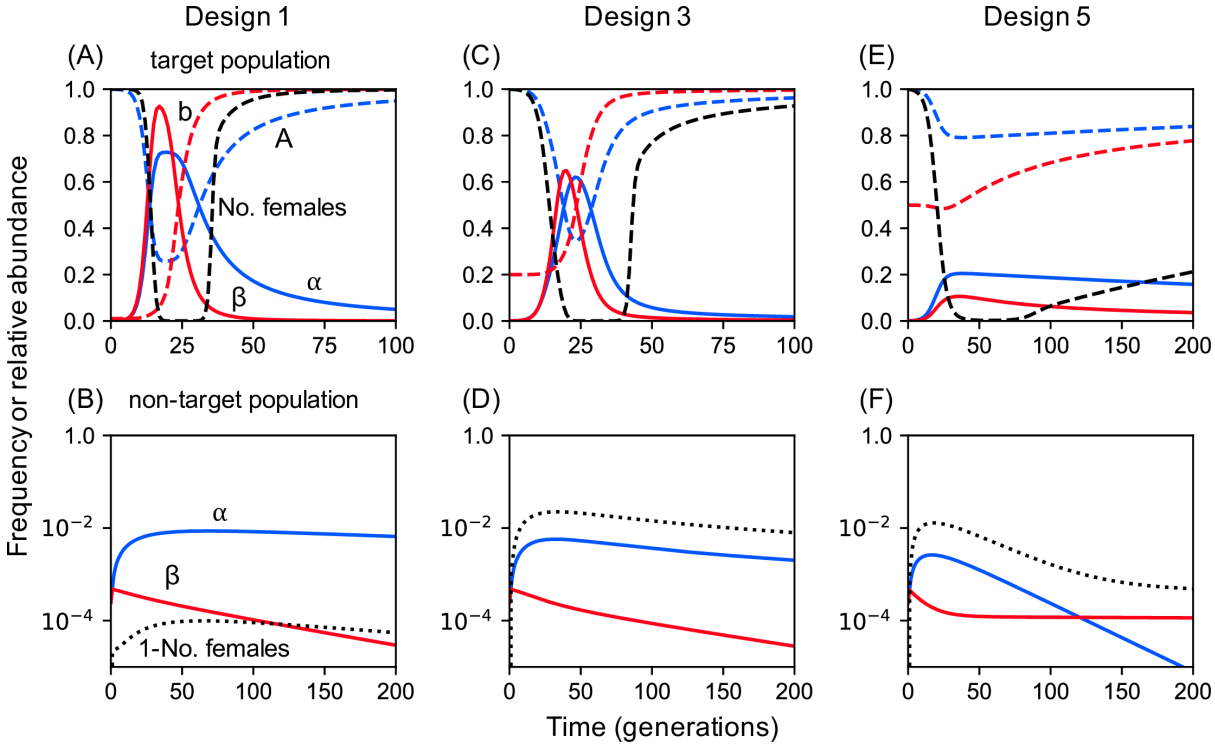


Fig 2. Performance of double drives for population suppression. (A, B) Timecourse for Design 1 in target and non-target populations, assuming 1% and 100% pre-existing resistance at the B locus, respectively. In the target population the α and β constructs increase in frequency together (blue and red solid lines), causing the number of females to decline. If the population is not eliminated, then eventually the resistant b allele replaces β, followed by the wild-type A allele replacing α, allowing the population to recover. In the non-target population both constructs remain rare and the reduction in female numbers remains small. **(C, D)** Timecourse for Design 3 assuming 20% and 100% pre-existing resistance in the target and non-target populations. **(E, F)** Timecourse for Design 5 assuming 50% and 100% pre-existing resistance in the target and non-target populations.

Because the spread of construct α in the target population depends on β, and therefore will be affected by the association between them, it might be expected that close linkage between the two constructs may increase construct spread and the extent of population suppression. Furthermore, because the population may eventually recover due to the evolution of resistance at the differentiated locus, additional improvements might be expected by using an essential gene as the differentiated locus and designing β to have minimal fitness effects (e.g., by containing a recoded version of the target gene [23-25], or being inserted in an artificial intron [26]). With such a design end-joining repair will tend to produce non-

functional resistance alleles, and resistance will be slower to evolve, relying instead on pre-existing resistant alleles. Both these expectations about linkage and using an essential differentiated gene are met individually, and, in combination, can reduce the minimum population size achieved by many orders of magnitude (Fig 3; see also S1 Fig for the separate effect of each modification). If it is not possible to have close linkage, then the maximum level of suppression can also be increased by releasing the two constructs in different males rather than in the same males, though at the cost of the impact being delayed, and separate releases perform worse than combined releases when linkage is tight (S2 Fig).

Design 2, which has the same components as Design 1, but arranged differently such that homing of the β construct only occurs in the presence of α, has dynamics qualitatively similar to Design 1, but quantitatively different (S3 Fig). Interestingly, if the two constructs are unlinked then the extent of suppression is less than with Design 1, but if they are closely linked then the suppression can be greater (Fig 3 and S1 Fig). All these possible alternatives give significantly greater suppression than a single drive imposing a reproductive load by targeting a female-essential gene (Fig 3).

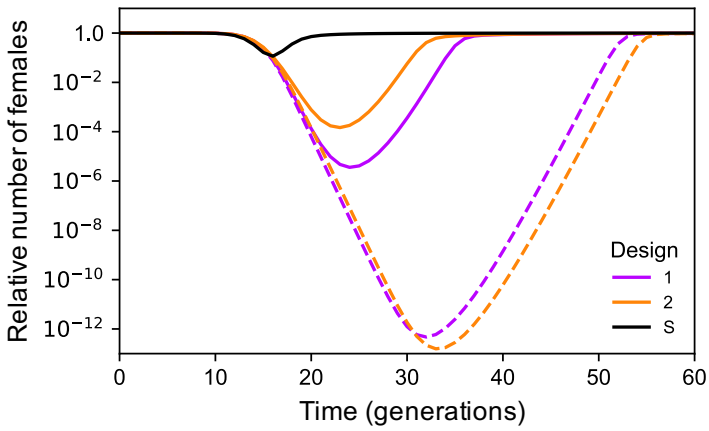


Fig 3. Timecourse for the relative number of females over time for Designs 1 and 2. Solid lines are for β in a neutral locus unlinked to the α construct, and dashed lines for β as a neutral insertion in a haplo-insufficient essential gene closely linked (r=0.01) to the α construct. In all

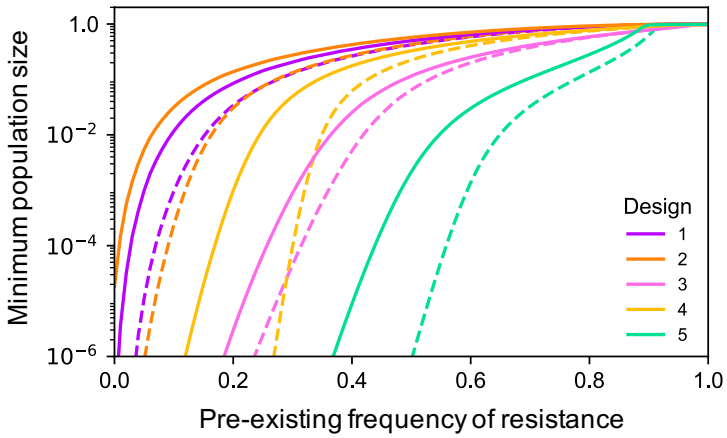
189 cases there is 1% pre-existing resistance at the B locus. Also shown for comparison are results
190 for a single construct drive targeting a haplo-sufficient female-specific viability gene (S).
191

192 **Coping with higher frequencies of pre-existing resistance**

193 Though these simple double drive designs work well with pre-existing target site
194 resistance at the differentiated locus of 1%, performance declines rapidly after that. For
195 example, if there is 10% pre-existing resistance, then even the best of these designs (Design
196 2 with close linkage and the differentiated locus being haplo-insufficient) only suppresses the
197 target population to a minimum of 2.38e-4. In some situations the target population may not
198 have a private allele with frequency over 90% and alternative approaches would need to be
199 considered. One possibility is to increase the load imposed on the population by the α
200 construct by adding to it an X-shredder locus that destroys the X-chromosome during
201 spermatogenesis such that it produces a male-biased sex ratio as well as killing heterozygous
202 females (Fig 1, Designs 3 and 4). Since population productivity in many species depends on
203 the number of females, population size may thereby be further reduced. A single drive based
204 on these components has previously been constructed in *Anopheles gambiae* by Simoni et al.
205 [27]. Our modelling indicates that adding an X-shredder to a double drive gives a quantitative
206 improvement in the dynamics, and even pre-existing resistance frequencies of 20% are
207 compatible with good control, while still having minimal effect on non-target populations (Fig
208 2C and D).

209 Even more robust control can be obtained by adding the X-shredder to the β locus and
210 having the α locus drive autonomously in males and cause dominant sterility or lethality in
211 females (e.g., target a female-specific haplo-insufficient locus; Fig 1, Design 5). The dynamics
212 in this case are somewhat different from the others: the X-shredder does not function to
213 directly increase the load, but instead it allows the α construct to spread in the population,
214 because it will end up more often in males (where it homes), and less often in females (where
215 it is a dead end). The male bias also protects the β construct from the female lethality produced
216 by the α construct, and so selection against β is much weaker than in the previous designs,

217 and resistance evolves more slowly (compare the rate of spread of the resistant b allele in Fig
218 2E to that in Fig 2A and C). As a result the design is able to perform well even with pre-existing
219 resistance of up to 50%, but still not spread in the non-target population (Fig 2E and F).
220 Moreover, if the population is not eliminated, it can nevertheless be suppressed for many
221 generations. For example, with 50% pre-existing resistance the minimum population size
222 reached is 2.15e-3, and the population remains below 5% of its pre-intervention size for 63
223 generations; with close linkage (r=0.01), then the corresponding values are 8.79e-7 and 147
224 generations. A comparison of the maximum extent of suppression as a function of the pre-
225 existing resistance frequency for the different designs is shown in Fig 4 (see also S4 Fig). With
226 all the modifications considered (linkage, use of an essential differentiated gene, or separate
227 releases) effects on the non-target population remain small (S5 Fig).



228

229 **Fig 4. Minimum population size for each of the 5 designs as a function of the pre-**
230 **existing frequency of resistance.** Solid lines are for the baseline case (r=0.5, β in a neutral
231 locus), while dashed lines are for the improved case (r=0.01, β as a neutral insertion in a
232 haplo-insufficient essential gene).
233

234 **Evolutionary stability and impact of fitness costs**

235 We now explore the consequences of relaxing two assumptions that have been implicit
236 thus far in our modeling. First, we have assumed that our various constructs remain intact
237 after release. In fact, mutations that destroy the function of one component or another will be
238 expected to arise as the constructs spread through a population, particularly as homing may
239 be associated with a higher mutation rate than normal DNA replication [28]. For components

that contribute directly to their construct's spread, one would expect that loss-of-function mutations would remain rare in the population and have little effect, whereas for other components (e.g., the X-shredder), such mutations may be actively selected for. To investigate we allowed homing-associated loss-of-function mutations to occur in each component of each construct. Mutation rates of 10e-3 have a small but significant impact on the performance of the three designs with an X-shredder, due to the accumulation of mutant constructs missing that component, while mutation rates of 10e-4 have negligible impact for all designs (Fig 5A, S6 Fig and S7 Fig).

Second, we have assumed thus far that the genetic constructs have little unintended impact on survival or reproduction. Experiments with *An. gambiae* have revealed at least two unintended fitness costs can occur, a reduced fitness of homing heterozygous females due to somatic expression of the nuclease [21, 27], and reduced fitness of males expressing an X-shredder, possibly due to paternal deposition of the nuclease and/or reduced sperm production [29]. The first of these costs is not relevant to Design 5 (because heterozygous females die anyway), and the second is not relevant to Designs 1 and 2 (because they do not use an X-shredder), but in other contexts, as expected, these costs reduce performance, requiring a lower frequency of pre-existing resistance in order to achieve a particular level of suppression (Fig 5B and C).

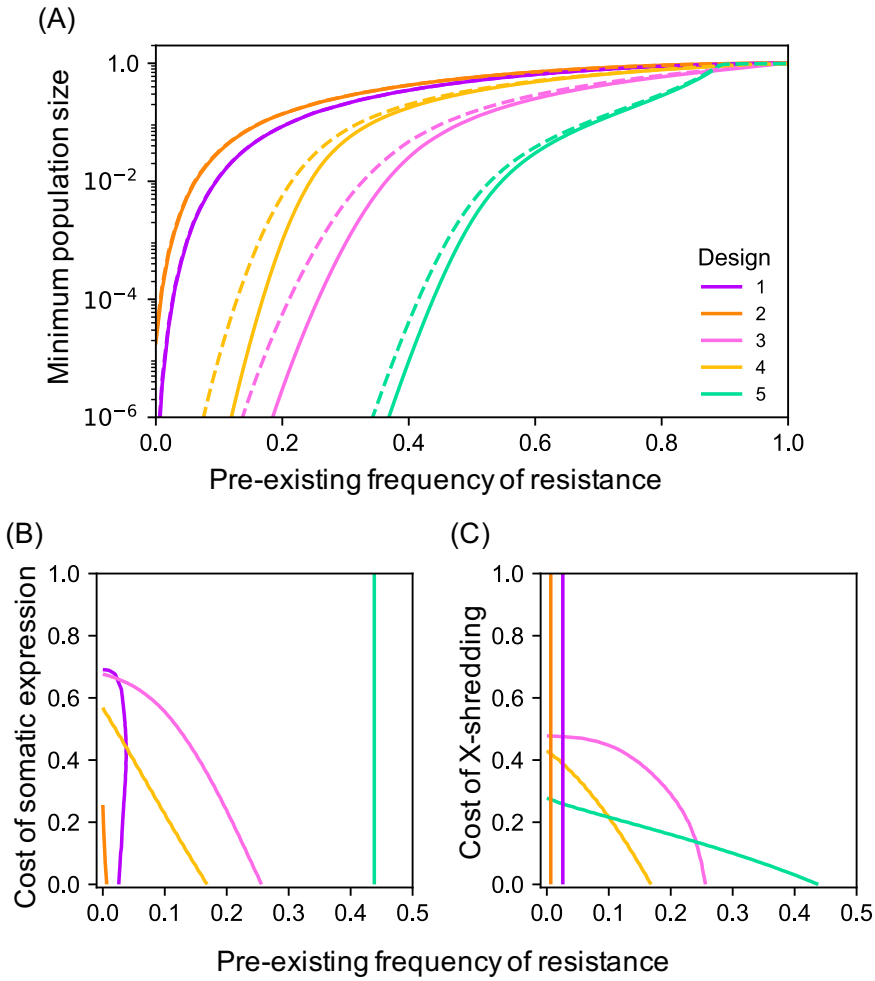


Fig 5. The impact of evolutionary stability and added fitness costs on the performance of each of the 5 designs for population suppression. (A) The effect of loss-of-function mutations on the minimum population size reached. Solid lines are for the baseline case of no mutations, and dashed lines are with each component of each construct having a mutation rate of 10e-3 per homing event. Note that the effect is only visible for designs with an X-shredder, and if the mutation rate was 10e-4, the results for all designs would be virtually indistinguishable from the solid lines. **(B, C)** Contour plots showing combinations of fitness costs and pre-existing frequency of resistance giving a minimum population size of 10e-4 for different double drive designs. For **(B)** the costs are reductions in female fitness due to somatic expression of the nuclease targeting the A locus, and for **(C)** the costs are reductions in male fitness due to the X-shredder. Vertical lines indicate the cost is irrelevant, either because heterozygous females in any case have fitness 0 (Design 5 in **(B)**), or because the designs do not include an X-shredder (Designs 1 and 2 in **(C)**).

Population replacement

Gene drive can be used not only for population suppression but also to introduce a new desirable 'cargo' gene into a target population for population replacement or modification – for example, a gene reducing a mosquito's ability to transmit a pathogen [30, 31]. In double drive designs for population replacement the α construct would carry the cargo and homing

by α would require β, while that by β could be either autonomous or depend on α (Fig 6A). Both α and β could be inserted into neutral sites, or into essential genes in such a way as to minimise fitness effects. We have modeled these approaches assuming, for purposes of illustration, the cargo imposes a dominant 20% fitness cost on females, and find that, again, such double drives can spread rapidly through target populations even when there is significant pre-existing resistance, and would not spread in non-target populations fixed for the resistant allele (Fig 6B and C). Unless there is virtually no pre-existing resistance at the differentiated locus, double drives can keep the frequency of the cargo gene above 95% much longer than a single drive construct targeting a differentiated locus, either neutral or essential (Fig 6D). If α is inserted in an essential gene, protection can be even longer.

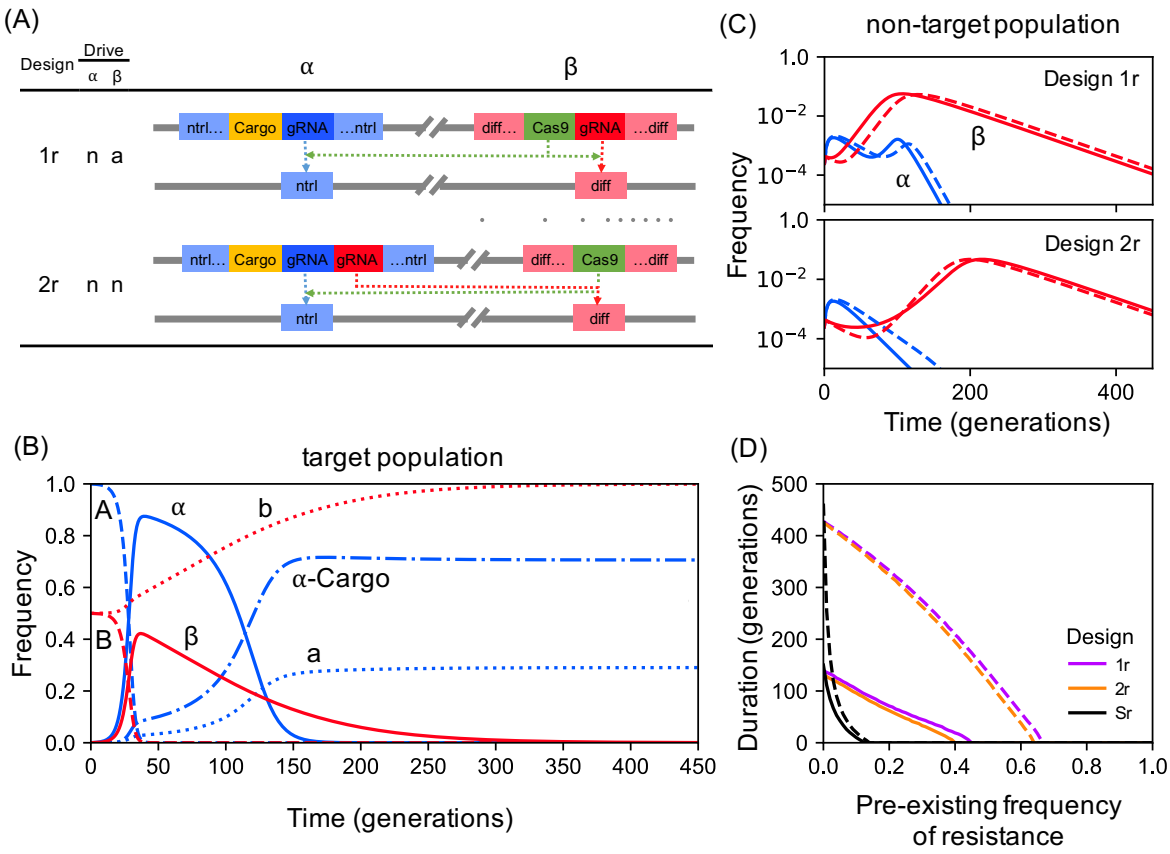


Fig 6. Double drives for population replacement. (A) Alternative double drive designs. **(B)** Timecourse of allele frequencies for Design 2r in a target population assuming 50% pre-existing resistance. The dynamics for Design 1r are qualitatively similar. **(C)** Allele frequencies for Designs 1r and 2r in a non-target population with 90% pre-existing resistance, assuming insertion of both *α* and β into neutral loci (solid lines); or *α* as a neutral insertion into a haplo-insufficient essential gene (dashed lines). **(D)** Duration of at least 95% of adult females carrying the cargo-bearing *α* construct as a function of the pre-existing frequency of resistance

296 at the B locus for double drives 1r and 2r, where solid and dashed lines are as in **(C)**. Also
297 shown for comparison are results for a single drive (S) carrying the cargo at a neutral A locus
298 (solid line) or a haplo-insufficient essential gene (dashed lines). Note that results for insertion
299 of β into a haplo-insufficient essential gene would be virtually indistinguishable from the solid
300 lines **(C, D)**. All plots assume 20% fitness cost of the cargo on females and a homing-
301 associated loss-of-function mutation rate of 10e-3.
302

303 **PAM site analysis in An. gambiae**

304 To explore whether the type of population differentiation assumed in our modelling can
305 exist in nature, we analysed published genome sequence data on *An. gambiae* mosquitoes
306 from the Ag1000G project [32]. The Ag1000G dataset includes sequences from 16 mainland
307 African populations and from populations on Mayotte and Bioko, two islands 500km off the
308 east and 30km off the west coast of Africa, respectively. Note that in presenting this analysis
309 we are not advocating the use of double drives on these islands, and merely wish to investigate
310 whether the requisite differentiation can be found on island populations. For our analysis we
311 focussed on potential PAM sequences (NGG or CCN), on the logic that a construct would be
312 unlikely to mutate to recognise a new PAM, whereas this could occur for a protospacer. The
313 entire dataset includes 57 million polymorphic sites, which we screened for PAM sites present
314 in the island population and at a frequency <10%, <5%, or absent from all other populations.
315 In Mayotte, for PAM sequences that were completely private to the island (i.e., not found in
316 any other population), only 1 of them had no pre-existing resistance (i.e., was found in all 48
317 sequences from the island), whereas 25 had pre-existing resistance less than 20%, and 353
318 had pre-existing resistance less than 50%. PAM sequences with small but nonzero
319 frequencies on the mainland were even more abundant (Fig 7). Bioko island is not as
320 differentiated as Mayotte from the mainland populations, and the sample size is smaller (18
321 sequences), but still there are some potential candidate sites.

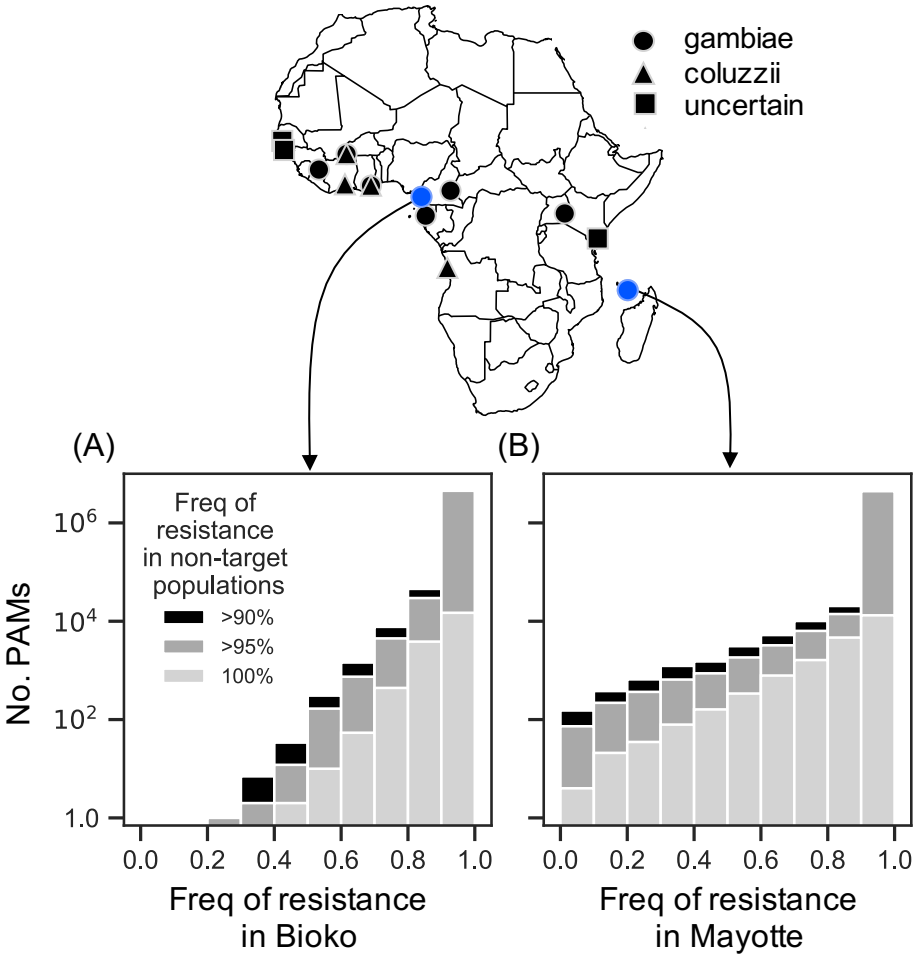


Fig 7. Frequency of PAM sites in island populations of An. gambiae. Numbers of PAM sites (NGG or CCN) with varying frequencies of resistance in samples of *An. gambiae* from two oceanic islands (blue map labels): **(A)** Bioko island (n = 18 sequences, from 9 individuals), and **(B)** Mayotte island (n = 48 sequences, from 24 individuals), where the PAM site frequency in each non-target population (black map labels) is <10%, <5% or 0% (i.e., target site resistance is >90%, >95% or 100%). GG or CC dinucleotides which varied by at least one base were considered to be resistant.

Discussion

Given that some of the most promising gene drive approaches for population control use (CRISPR-based) sequence-specific nucleases, an obvious way to limit their spread and impact is to exploit sequence differences between target and non-target populations. In this paper we have proposed using a double drive design, here defined as one that uses two constructs, inserted at different locations in the genome, both of which can increase in frequency, and which interact such that the transmission of at least one of them depends on

338 the other. Previously published examples that fit this definition include those for 2-locus under-
339 dominance [14, 15, 33, 34], and Medusa [35], tethered [36], integral [26], and
340 transcomplementing [37] gene drives. As with single-construct gene drives, these various
341 proposed designs differ in purpose (suppression vs. modification), release rate needed to
342 initiate spread (low vs high threshold), and the molecular basis for the superMendelian
343 inheritance (homing, toxin-antidote interactions, or a combination of the two). The requirement
344 that both constructs can increase in frequency over time excludes split drives [25, 38-40] and
345 killer-rescue systems [41, 42], in which only one of the two components increases in
346 frequency. In our proposed designs there is a division of labour between the two constructs,
347 with one responsible for the desired impact (suppression or replacement) and the other for the
348 population restriction, such that together they act as a double drive in the target population
349 and as a split drive in non-target populations. Note that if there are multiple populations of the
350 same species requiring control, each with a different private allele, the same α construct could
351 be used in each case, with only a change in the insertion site of the β construct and the
352 corresponding gRNA. This flexibility may be particularly useful when the α construct requires
353 significant optimisation [26].

354 We have considered a range of double drive designs of increasing resilience, as
355 judged by their ability to cope with an increasing frequency of pre-existing resistance at the
356 differentiated locus. The simplest designs do not have any component beyond those needed
357 for any CRISPR-based construct, and so should be widely applicable [37]. More powerful
358 constructs can be made by adding an X-shredding sex ratio distorter to the load-inducing
359 construct; these have been most effectively demonstrated in *An. gambiae* mosquitoes [11,
360 43], but may also work more broadly [44]. In other species there are other ways to distort the
361 sex ratio [45-47], and it would be interesting to model whether these alternatives would be
362 expected to have the same impact as an X-shredder in the context of a double drive. An even
363 simpler way to increase the load would be to add extra gRNAs to the α construct that mutate
364 other female fertility genes [48, 49]. The most powerful design we considered targets a female-

365 specific haplo-insufficient gene, or otherwise causes dominant female sterility or lethality.
366 Such genes are not common, but there are some possible candidates [50-52], and our
367 modelling motivates the search for others. Finally, performance (in terms of being able to cope
368 with ever higher frequencies of pre-existing resistance) could presumably also be improved
369 by using a third construct, to construct a triple drive, though modelling would be required to
370 explore the implications of the many different configurations this extension would allow.

371 The proposed strategy requires that there be a differentiated locus between target and
372 non-target populations. It need not be an essential gene, and could even be selectively neutral.
373 Our focus has been on using so-called private alleles – sequences that are present (but not
374 necessarily fixed) in the target population, and absent (or of negligible frequency) in non-target
375 populations. Our analysis of PAM sites in *An. gambiae* indicates that appropriately
376 differentiated sites may exist in island populations of this species, though our analysis must
377 be considered preliminary: the dataset does not include mainland sites in closest proximity to
378 the island populations, where differentiation may be lower, and we have not considered
379 potential polymorphism in the protospacer sequence (which, if present, may require the use
380 of multiple gRNAs). We have focussed on nucleotide variation at PAM sites on the assumption
381 that a construct is unlikely to mutate to recognise a new PAM; structural variation in the
382 protospacer region may also be an appropriate basis for geographically restricting double drive
383 spread. We have also not attempted to determine whether the observed differentiation is due
384 solely to mutation and drift, or if selection may be involved as well.

385 Note that the single drives modelled by Sudweeks et al. [19] require the opposite type
386 of differentiation: sequences that are fixed in the target population, even if not private (i.e.,
387 even if found at appreciable frequencies in the non-target population). In this latter scenario
388 the challenge is not so much to have an impact on the target population as to not have an
389 impact on the non-target population. What constitutes "acceptable non-impact" may differ
390 widely from one use case to another and must be assessed on a case-by-case basis: in some
391 circumstances spread of the construct and a transient decline in population size followed by
392 recovery may be acceptable, whereas in others any significant spread of the construct may

be unacceptable, regardless of impact on population size. Designs with non-autonomous homing of the β construct (Designs 2, 4, and 5) should be less likely to increase in frequency in the non-target population, and may therefore be preferable. We have focused in this paper on differentiated loci on autosomes, but note that for Design 5 the X-shredder is required for the spread of the α construct and, in principle, one could achieve population-restricted spread if the shredder targeted a population-specific sequence on the X chromosome (rather than inserting it into a population-specific autosomal sequence). In many species the X chromosome shows greater population differentiation than autosomes [53], so this alternative may be useful. Finally, if there are no private alleles in the target population, it may be worthwhile considering a two-step approach of first introducing a private allele into a population and then using that allele to control the population [18]. The ability of double drives to exploit private alleles that are selectively neutral and that have a frequency of only 50% (suppression) or 20% (modification) potentially makes this approach more feasible than would otherwise be the case.

In this paper we have used a simple high-level modelling framework in which the generations are discrete, the population is well mixed, and dynamics are deterministic. This framework is appropriate for strategic models aiming to identify candidate approaches that are worthy of further investigation. For any specific use case the appropriate tactical models would need to be developed that incorporate more biological detail, including spatial and stochastic effects. Such extensions will be particularly important when the goal is to eliminate the target population, which is not possible in our deterministic models. Instead, we have reported the minimum relative population size achieved, which is expected to be related to the size of a population that could be eliminated, but determining the precise connection will require bespoke modelling tailored to a specific situation. Further extensions would be needed to allow for on-going movement between target and non-target populations – if there is on-going immigration into the target population, and this cannot be stopped, then it may not be possible to eliminate the target population with a single release of a double drive. Nevertheless, such

420 a release may be sufficient to suppress the population to such an extent that it can be
421 controlled by other means, including recurrent releases of the same constructs. If one is able
422 to achieve an initial release rate of 1% into a target population, and that suppresses the
423 population by a factor of 1000, then the same releases going forwards will constitute a 10-fold
424 inundation, and self-limiting genetic approaches may be sufficient.

425 Methods

426 The basic modelling structure follows that of Burt & Deredec [10]. In brief, populations
427 have discrete generations, mating is random, there are two life stages (juveniles and adults),
428 and juvenile survival is density dependent according to the Beverton-Holt model, which has
429 two parameters, but since we report results in terms of relative population sizes, only one
430 matters, the intrinsic rate of increase (R_m). We assume this is equal to 6 [49]. Genetic
431 parameter values (rates of DNA cleavage, rates of alternative repair pathways, and the sex
432 ratio produced by X-shredding) are as estimated from *An. gambiae* [11, 21, 54]. Constructs
433 may be inserted into a haplo-sufficient or haplo-insufficient female-essential gene (in which
434 case gene function is disrupted), a selectively neutral sequence (in which case the insertion
435 is also selectively neutral), or a haplo-sufficient or haplo-insufficient gene required for male
436 and female viability (in which case the insertion is again selectively neutral, because it contains
437 a re-coded version of the target gene [23-25], or is inserted in an artificial intron [26]). For
438 constructs inserted into an essential gene we assume end-joining repair produces non-
439 functional cleavage-resistant alleles [21, 55], while for constructs inserted into selectively
440 neutral sites the products of end-joining repair are also neutral. In all models we assume
441 individuals with an intact CRISPR system suffer a 1% fitness cost for every different gRNA
442 they carry as a cost of off-target cleavage, and for population replacement we assume the
443 cargo gene imposes a 20% fitness cost on females. Both these costs are assumed to be
444 dominant. For simplicity, we assume all fitness costs affect survival after density dependent
445 juvenile mortality and before censusing (e.g., as if pupae die). All results are for populations
446 censused at the adult stage. Releases are of heterozygous adult males at 0.1% of the pre-

release number of males, and if the two constructs are linked then they are assumed to be in cis; for constructs released in separate males we assume release rates of 0.05% of each. A list of parameters and their baseline values is given in S1 Table and S2 Table. For the PAM site analysis we screened the Ag1000G phase II SNP data for PAM sites (GG or CC dinucleotides) showing variation between samples at one or both nucleotides. PAM site frequencies were calculated per sampling location and filtered for those present in the island population and at <10%, 5%, or absent from all other populations, excluding those containing >5% missing data in at least one sampled population. Further details are given in the S1 Appendix.

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Supporting Information

S1 Text. Supplemental methods.

Genetics and fitness effects of disrupting host genes

Our baseline model has 2 autosomal loci each with 3 alleles. At the first locus A is the wildtype allele; α is the transgenic construct inserted into and disrupting the wild type allele; and a is a cleavage-resistant allele produced by end-joining repair. For population suppression we assume that the A locus is needed for female survival past the pupal stage, that the resistant allele is non-functional, and that functional resistance is not possible [1]. When A is a haplo-sufficient gene, we assume no reduction in survival probability when carrying only one functional copy of the gene. At the second locus there are initially 2 segregating alleles of equal fitness, B and b, with B being more common in the target population than in the non-target population (and vice versa for b). The third allele, β, is the transgenic construct which can home into B but not into b. Where the differentiated B locus is neutral, we assume all end-joining repair events produce cleavage-resistant alleles (b). In the case where the differentiated B locus is an essential gene, we extend the model to have a 4th allele at this locus, bj, which is a non-functional, cleavage resistant, version of B that can be formed by end-joining repair and assume all end-joining leads to non-functional alleles. We also assume that the β construct has been engineered to have minimal effects on fitness (e.g., by having a recoded version of the B allele, or being inserted into an artificial intron). For comparison we also model a single drive consisting of a single construct homing into a female viability gene, and allow for both functional resistance genes that may pre-exist in the population and non-functional resistance alleles created by end-joining (4 allele model; Fig 3 in main text).

For population replacement where α carries a desirable cargo gene and β is in the differentiated locus, we consider three cases, where (i) both A and B loci are selectively neutral; (ii) the A locus is selectively neutral, and B is a haplo-insufficient essential gene; or (iii) the A locus is a haplo-insufficient essential gene, and the B locus is selectively neutral. In those cases where the A or B locus is an essential gene, the α or β construct is assumed to

665 have been designed to have minimal effects on fitness (as above), end-joining produces non-
666 functional alleles which are dominant lethal, and any pre-existing resistant allele (at B) is
667 selectively neutral. In each case there are 3 alleles at each locus, except when B is an
668 essential gene, when there are 4. For comparison we also consider a single drive consisting
669 of a construct that homes into a neutral site (3 allele model) or into a haplo-insufficient
670 essential gene (4 allele model; Fig 6 in main text).

671 Where the fitness of either A/A or B/B homozygotes is standardised to 1, the fitness of
672 individuals homozygous for the construct α/α or β/β is $1 - s_I$; homozygous for the cleavage
673 resistant allele a/a or b/b is $1 - s_R$; heterozygous for the construct A/α or B/β is $1 - h_I s_I$;
674 heterozygous for the cleavage resistant allele A/a or B/b is $1 - h_R s_R$ or heterozygous carrying
675 both the construct and cleavage-resistant allele α/a or β/b is $1 - s_{IR}$, where s_I, s_R and s_{IR} are
676 selection coefficients and h_I and h_R are dominance coefficients, each of which differ
677 depending on the locus (A and B) and between sexes (see S2 Table for more details of locus-
678 specific fitness costs for each scenario modelled).

679 ***Construct activity and fitness effects***

680 Each construct consists of one or more transcription units (Cas9, gRNA, X-shredder,
681 Cargo), and the activity associated with each is assumed to be dominant. For individuals
682 carrying at least one Cas9, one gRNA and the site targeted by the gRNA (A or B), cleavage
683 of the target site occurs with probability c, after which end-joining repair occurs with probability
684 j converting the target site to a resistant allele (a or b), and homing occurs with probability $1 -$
685 j converting the target site to the construct (α or β). For males carrying at least one X-shredder,
686 sperm is produced carrying Y or X chromosomes at a ratio of $m: 1 - m$. In all models, we
687 assume a fitness cost due to off-target cleavage (sH) of 1%, where fitness is reduced by a
688 factor $1 - sH$ if there is at least one Cas9 and one gRNA present, or by a factor $(1 - sH)^2$ if
689 there is at least one Cas9 and two different gRNAs present. To model costs due to somatic
690 expression of the nuclease, individuals carrying at least one Cas9, one gRNA and the site
691 targeted by the gRNA, have fitness reduced by a factor $1 - sS$. Similarly, to model costs

associated with X-shredding, males carrying at least one X-shredder have fitness reduced by a factor $1 - sX$. With the exception of Fig 5B and C in the main text, we assume both sS and sX to be zero. For population replacement we assume the cargo has a fitness cost (sC) of 20% in females, with no effect on males. In all cases the fitness costs associated with each unit's activity are assumed to be dominant, and for simplicity, are assumed to affect survival of individuals after density dependence and before censusing (e.g., at the pupal stage).

The overall fitness of an individual is therefore the product of the relative fitness due to host gene disruption at locus A, host gene disruption at locus B and construct activity.

Analyses of evolutionary stability

We extend both the 3-allele and 4-allele models to allow for loss-of-function mutations of each transcription unit (Cas9, gRNA, X-shredder, cargo). After release of fully functional constructs, we assume that each unit mutates with probability μ; mutation can only occur during homing; it is possible for more than one unit to mutate during a single homing event; and mutation back to a functional unit does not occur. Constructs carrying non-functional units are expected to carry the fitness costs associated with disrupting the function of the host gene, however, no longer carry costs due to the transcription unit activity (e.g., off-target cleavage). Although at the sequence level constructs carrying only non-functional units are expected to differ from cleavage-resistant alleles produced through end-joining repair, both are assumed to be resistant to cleavage and carry the same fitness costs, therefore we model them as a single allele.

Population biology

We model a population with discrete, non-overlapping generations. In each generation, we assume the population mates randomly, that females produce f fertilised eggs and that males are not limiting in their production. Juvenile survival is density-dependent, such that the probability of surviving is $\theta_J \frac{\gamma}{\gamma + N_J}$, where θ_J is the density-independent probability the juvenile survives to adulthood, γ determines the strength of density dependent mortality and N_J is the total number of juveniles in the population during the generation. The intrinsic rate of increase

719 (R_m) of wild-type population is $\frac{f\theta_J}{2}$. After density-dependent juvenile mortality, additional
720 genotype-dependent mortality occurs before juveniles mature into adults, taking into account
721 fitness costs associated with the constructs and cleavage-resistant alleles. Adult males and
722 females then produce gametes, during which recombination occurs between the A and B loci
723 with probability r, and, depending on the genotype, cleavage followed by homing or mutation
724 may occur in males or females, and shredding of the X-chromosome may occur in males.

725 ***Population censusing***

726 Relative population densities and allele frequencies are of populations censused at the
727 adult stage and the correlation between the constructs α and β is:

728 $$\frac{(p_{\alpha\beta} - p_\alpha * p_\beta)}{\sqrt{p_\alpha(1-p_\alpha)p_\beta(1-p_\beta)}} \quad (1)$$

729 where, $p_{\alpha\beta}$ is the frequency of αβ chromosomes in the population and p_α and p_β are the
730 frequency of α and β alleles in the population respectively, each calculated from populations
731 censused at the adult stage.

732 ***PAM site analysis in An. gambiae***

733 To identify PAM sites (NGG or CCN) in *An. gambiae* with the type of differentiion
734 required for our approach, SNP variants from the Phase II Ag1000G dataset were first
735 screened for the presence of at least one G or C allele. Positions upstream and downstream
736 of the variant were then screened to calculate frequencies of GG and CC dinucleotides, using
737 the AgamP4 reference genome for invariant neighbouring sites, or the Ag1000G SNP data for
738 neighbouring variants. Since phased data was only available for biallelic variants,
739 dinucleotides containing two variants, of which at least one was multiallelic, were not screened
740 (24% of G or C variants). For each differentiated PAM site, frequencies were calculated for
741 the 16 sampled populations (Cameroon *An. gambiae* n=594, Uganda *An. gambiae* n=224,
742 Burkina Faso *An. gambiae* n=184, Guinea-Bissau uncertain sp. n=182, Angola *An. coluzzii*
743 n=156, Burkina Faso *An. coluzzii* n=150, Cote d'Ivoire *An. coluzzii* n=142, Gabon *An. gambiae*
744 n=138, The Gambia uncertain sp. n=130, Ghana *An. coluzzii* n=110, Kenya uncertain sp.

745 n=96, Guinea *An. gambiae* n=80, Mayotte *An. gambiae* n=48, Ghana *An. gambiae* n=24,
746 Bioko *An. gambiae* n=18 and Guinea *An colluzzi* n=8, where n is the number of sequences
747 and n/2 is the number of individuals sampled). PAM sites containing >5% missing data at any
748 sampling location were removed (0.5% PAMs), leaving a total of 13,462,450 polymorphic PAM
749 sites. The per-site PAM frequencies were used to identify PAM sites present in the island
750 population of interest, and at frequencies <10%, <5% or absent in all other populations,
751 excluding the *An. coluzzii* population from Guinea due to low sample size (n=8, from 4
752 individuals). Analysis of the Ag1000G data made heavy use of the python package scikit-allel
753 (https://doi.org/10.5281/zenodo.3238280).

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759

S1 Table. Model parameters and baseline values.

Parameter	Description	Baseline value
R_m	Intrinsic rate of population increase	6
r	Recombination between loci	0.5
d	Proportion of offspring carrying transgene	0.976 [1]
u	Proportion of non-homed chromosomes which are mutant	0.392 [2]
e	Probability of homing	$2\mathrm{d} - 1 = 0.953$
c	Probability of cleavage	$e + (1 - e)\mathrm{u} = 0.971$
j	Probability of end joining given cleavage	$\frac{(1-e)u}{e+(1-e)u} = 0.019$
m	Proportion of Y-bearing sperm produced by X-shredder males	0.95 [3]
s_H	Cost of off-target cleavage in individuals expressing Cas9 and gRNA (targeting either allele A or B) (dominant)	0.01
s_S	Female cost of somatic cleavage of wildtype allele by Cas9 and gRNA (targeting either allele A or B) (dominant)	0
s_X	Male cost of X-shredding (dominant)	0
s_C	Female cost of cargo expression	0.2
p	Probability of a functional mutant being produced through end joining	0
μ	Probability of each component becoming non-functional during homing	0

References

1. Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A, Beaghton AK, et al. A CRISPR-Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes. Nat Biotechnol. 2018;36(11):1062-6. Epub 2018/09/25. doi: 10.1038/nbt.4245. PubMed PMID: 30247490.

2. Hammond A, Karlsson X, Morianou I, Kyrou K, Beaghton A, Gribble M, et al. Regulation of gene drive expression increases invasive potential and mitigates resistance. Biorxiv. 2020.

3. Galizi R, Doyle LA, Menichelli M, Bernardini F, Deredec A, Burt A, et al. A synthetic sex ratio distortion system for the control of the human malaria mosquito. Nat Commun. 2014;5:3977. Epub 2014/06/11. doi: 10.1038/ncomms4977. PubMed PMID: 24915045; PubMed Central PMCID: PMCPMC4057611.

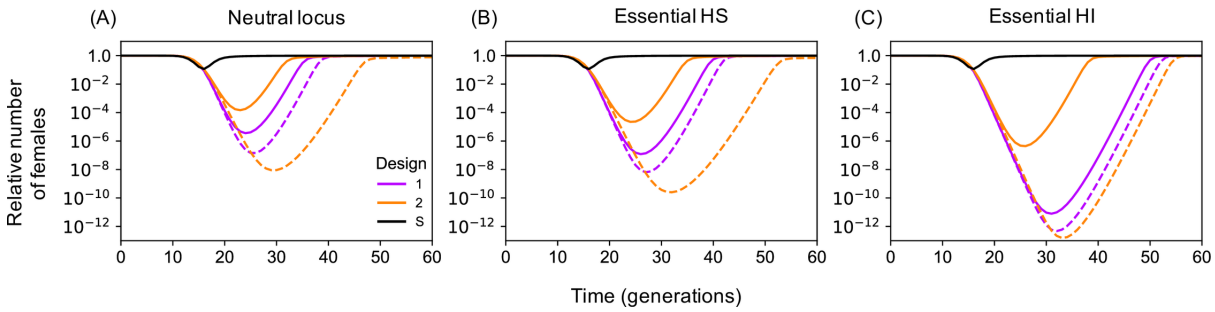
S2 Table. Host gene disruption fitness costs.

		A-suppression*		A-replacement or B†		
Parameter	**Description**	**Haplo-sufficient**	**Haplo-insufficient**	**Neutral**	**Haplo-sufficient**	**Haplo-insufficient**
s_I	Fitness cost for target site disruption by insertion of construct	1	1	0	0	0
h_I	Dominance coefficient for target site disruption by insertion of construct	0	1	0	0	0
s_R	Fitness cost for target site disruption by resistant allele	1	1	0	1	1
h_R	Dominance coefficient for target site disruption by resistant allele	0	1	0	0	1
s_{IR}	Fitness cost for heterozygotes (construct/resistant)	1	1	0	0	1

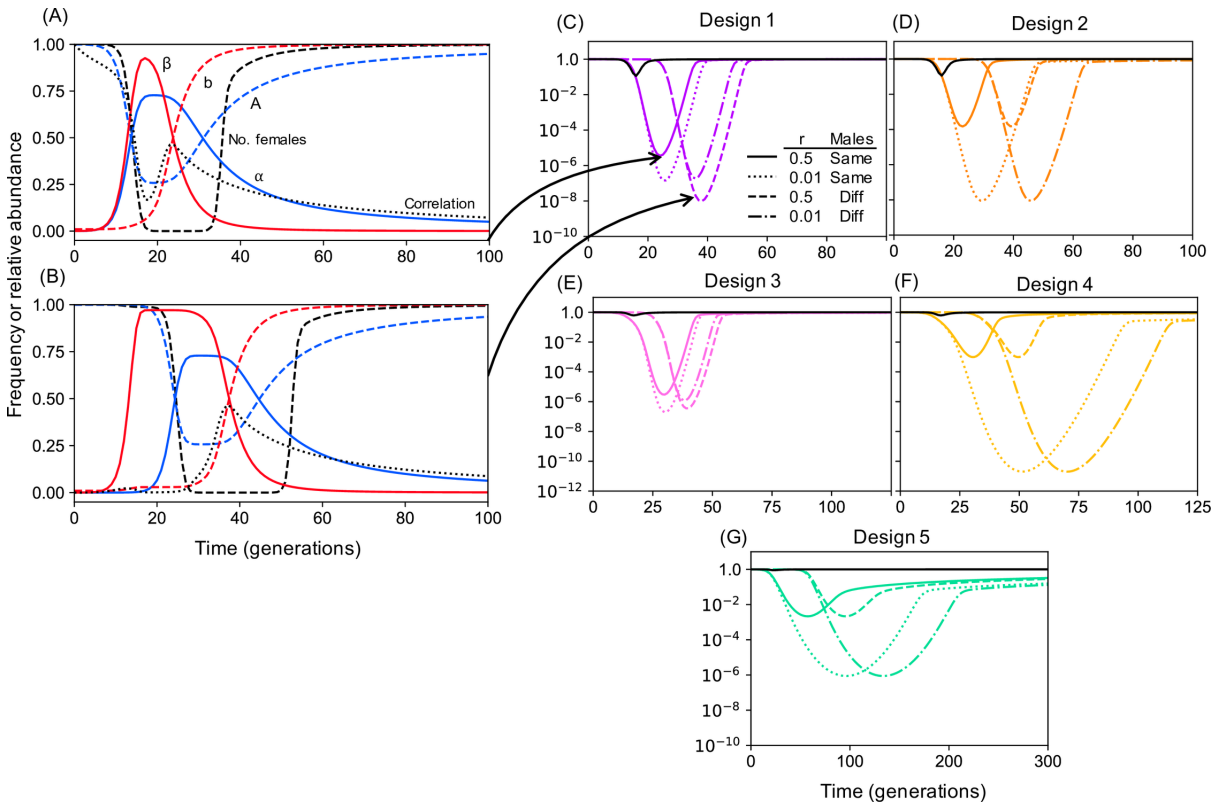
s_I, s_R and s_{IR} are selection coefficients associated with host gene disruption and h_I and h_R are dominance coefficients. At locus A in suppression double drives, fitness costs for disruption of function at the target site can occur either by insertion of the construct (I) or by end-joining that produces non-functional cleavage resistance (R), resulting in recessive or dominant female-specific lethality for haplo-sufficient or -insufficient genes respectively. At locus A in replacement double drives, or at the differentiated locus B in either suppression or replacement drives, fitness costs for disruption of the target site by construct insertion are assumed to be zero (e.g., by having the construct contain a recoded version of the target gene). Where the target site is a haplo-sufficient or -insufficient essential gene, non-functional cleavage-resistant mutants are expected to cause recessive or dominant lethality respectively, affecting in both males and females.

* Fitness costs are female-specific.

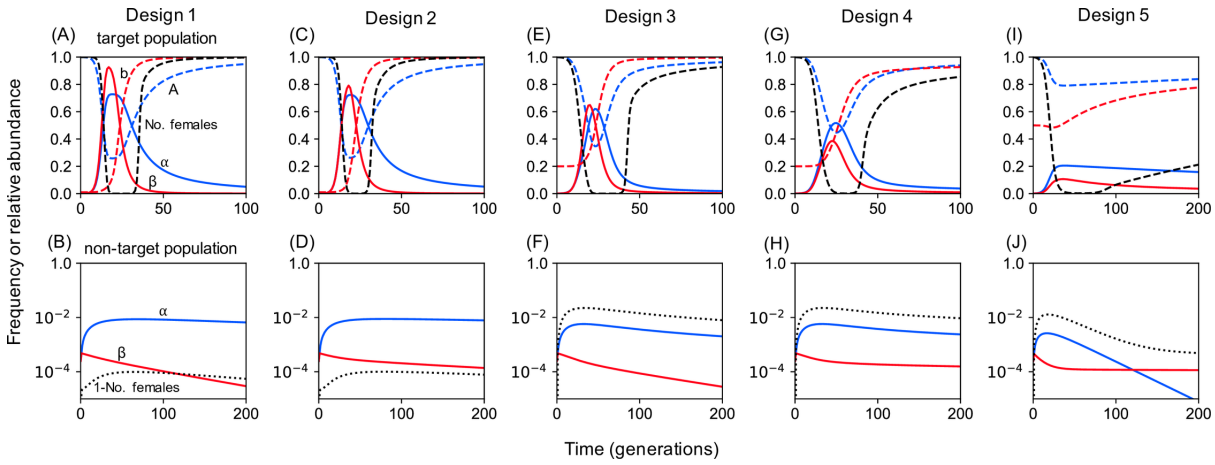
† Fitness costs apply to both males and females.



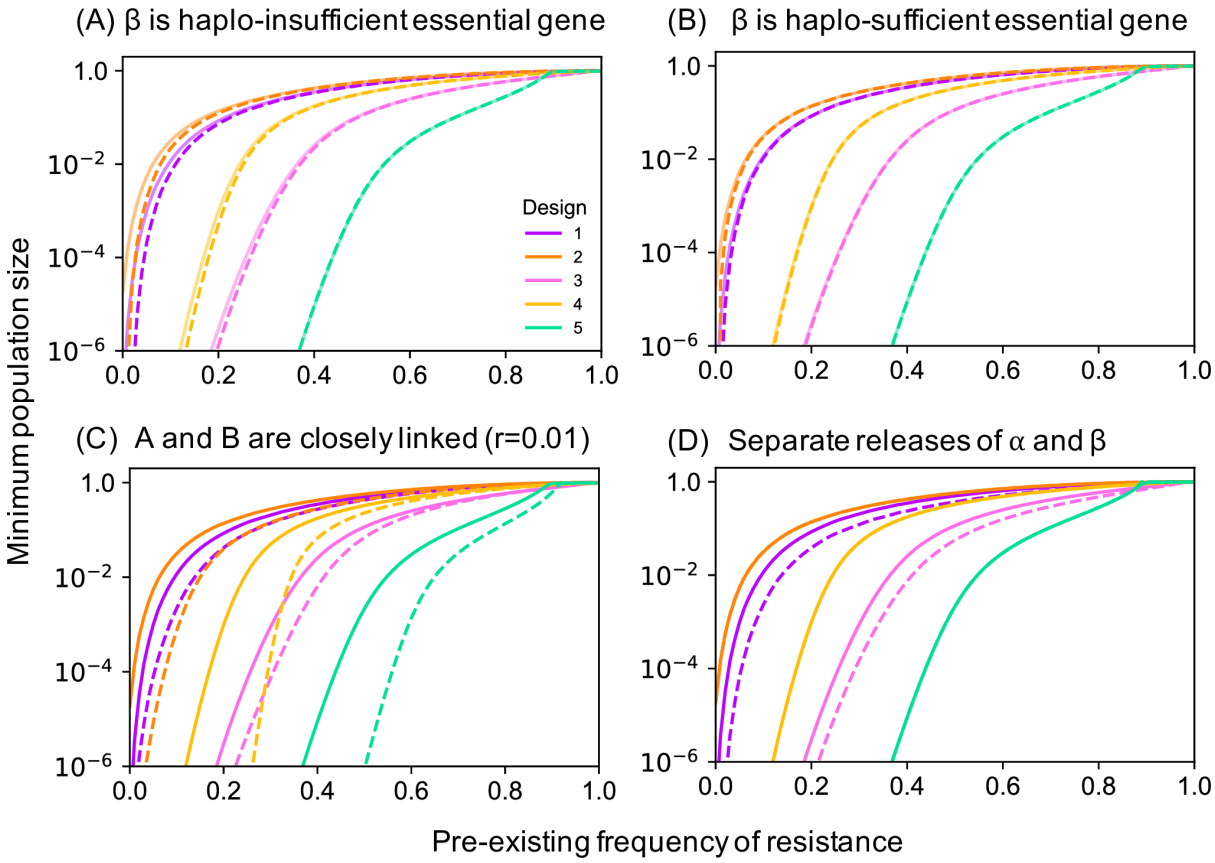
S1 Fig. Timecourse for the relative number of females over time for Designs 1 and 2. Solid lines are for where α and β are unlinked and dashed lines for where they are linked (r=0.01). Shown are the cases where β is inserted as a neutral insertion into **(A)** a neutral locus, **(B)** an essential haplo-sufficient gene or **(C)** an essential haplo-insufficient gene. Shown for comparison is a time course for a single drive targeting a haplo-sufficient female-specific viability gene (S).



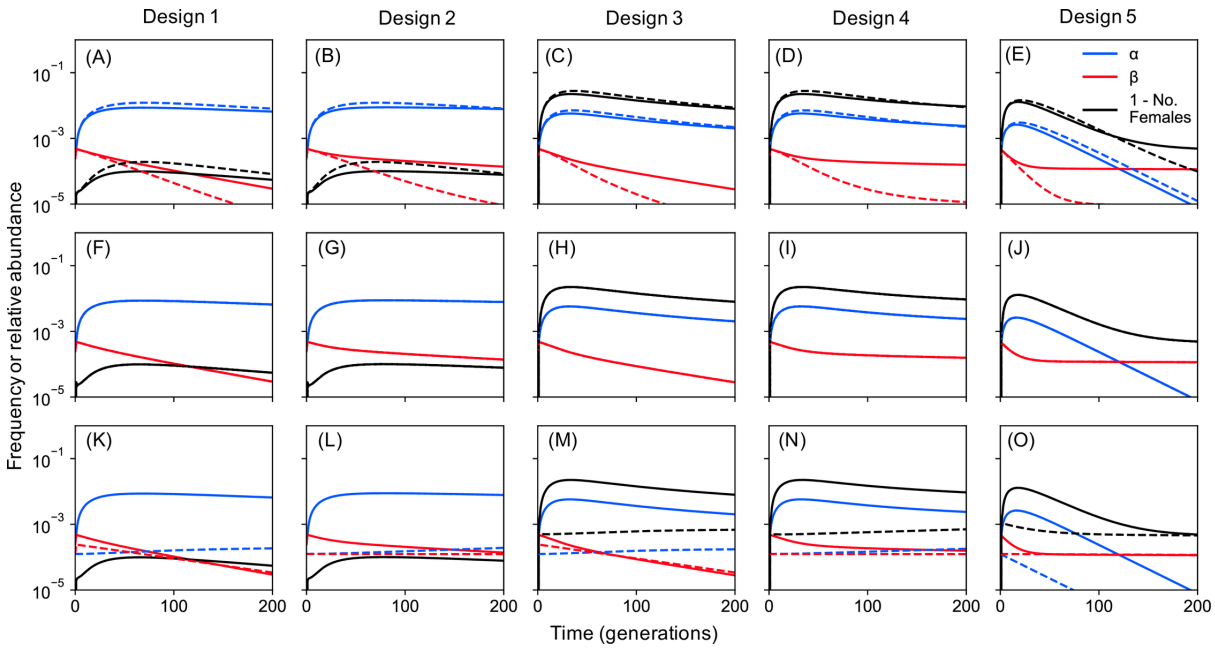
S2 Fig. Effect of releasing constructs in the same or different males. Comparison of dynamics for Design 1 when the constructs are unlinked and are released in the same **(A)** or in different **(B)** males. If the constructs are released in separate males the initial correlation between α and β (black dotted line) is negative, allowing β (solid red line) to increase to a higher frequency than if released in the same males as α where it experiences higher fitness costs. Consequently α (solid blue line) is retained at high frequency in the population for longer resulting in a greater reduction in relative number of females, though there is a longer delay between release and impact. **(C-G)** Timecourse for the relative number of females over time for Designs 1-5 where constructs are unlinked and released in the same males (solid lines), linked and released in the same males (dotted lined), unlinked and released in different males (dashed lines) or linked and released in different males (dot-dashed). Pre-existing resistance is assumed to be 1% **(C, D)**, 20% **(E, F)** and 50% **(G)**. For Designs 2, 4 and 5 **(D, F, G)** separate releases only delay the impact because β cannot increase in frequency autonomously, whereas for Designs 1 and 3 separate releases can give a larger (though still delayed) impact when constructs are unlinked, but not when they are closely linked. Shown for comparison is a time course for a single drive targeting a female-specific viability gene with the same level of pre-existing resistance (1%, 20%, or 50%; black solid lines).



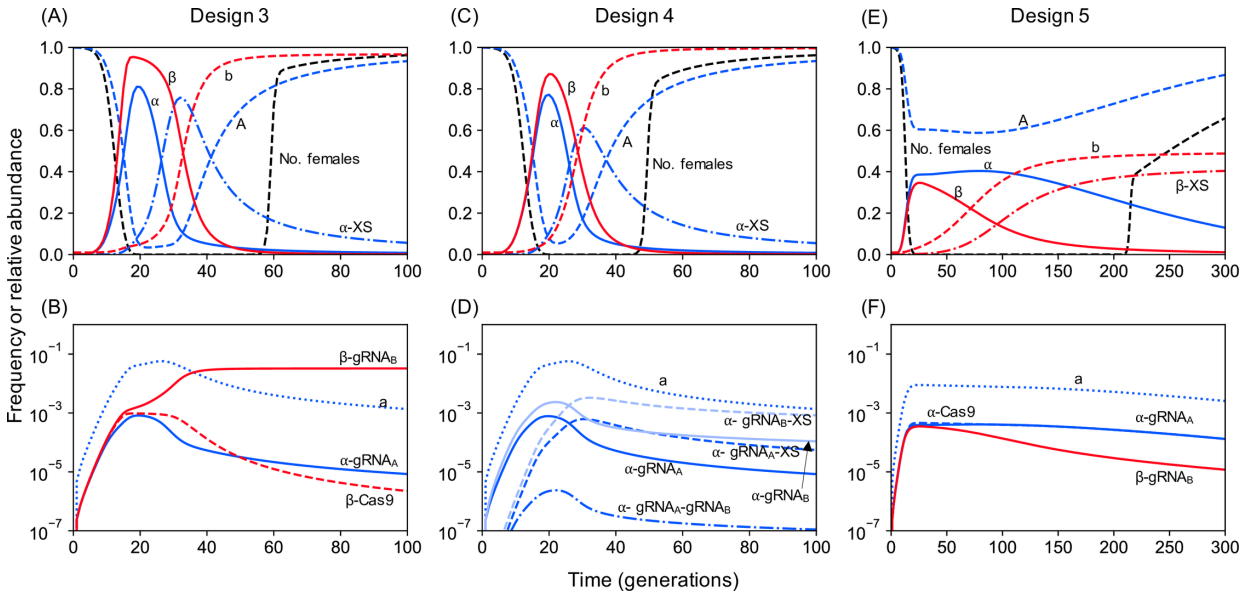
S3 Fig. Example time courses for double drives for population suppression. Design 1 **(A, B)** and 2 **(B, C)** assuming 1% and 100% pre-existing resistance in target and non-target populations. Design 3 **(E, F)** and 4 **(G, H)** assuming 20% and 100% pre-existing resistance in target and non-target populations. Design 5 **(I, J)** assuming 50% and 100% pre-existing resistance in target and non-target populations respectively. Plots for Designs 1, 3, and 5 are the same as in the main text, and presented here to facilitate comparisons.



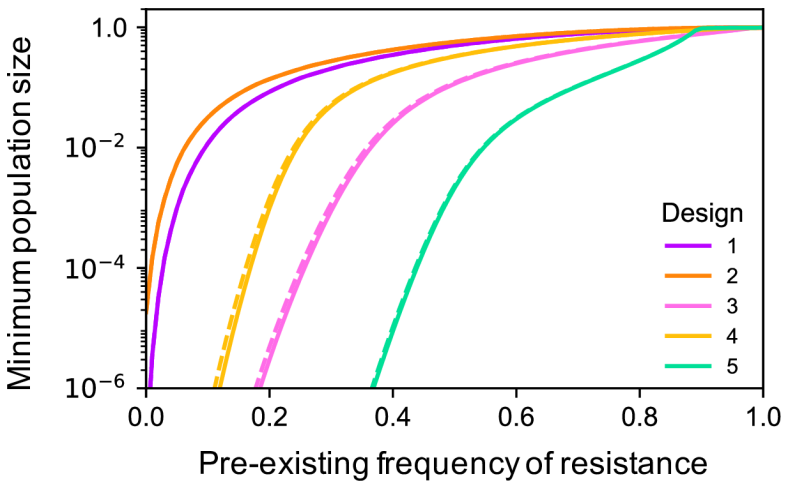
S4 Fig. The impact of alternative designs and their variants on population suppression as a function of the pre-existing frequency of resistance. Solid lines are for baseline conditions (α and β are released in the same males, β is in a neutral locus, and loci are unlinked), and are the same in each panel. Dashed lines are for variants where **(A)** β is inserted as a neutral insertion into an essential haplo-insufficient gene, **(B)** β is inserted as a neutral insertion into an essential haplo-sufficient gene, **(C)** loci are linked (r=0.01), and **(D)** α and β are released in separate males, holding all other properties at baseline.



S5 Fig. Timecourse of allele frequencies and population suppression (1-relative number of females) for Designs 1-5 in non-target populations where the resistant allele is present at 100%. (A-E) α and β are unlinked (solid lines) or linked (dashed lines). **(F-J)** β is inserted as a neutral insertion into a neutral site (solid lines), haplo-sufficient essential gene (dashed lines) or haplo-insufficient essential gene. **(K-O)** α and β are released in the same males (solid lines) or different males (dashed lines).



S6 Fig. Timecourses for Designs 3, 4 and 5 assuming pre-existing frequency of resistance of 1%, where homing-associated loss-of-function mutations occur for each component of each construct with probability 10e-3. For each design, the intact constructs (α, blue solid lines and β, red solid lines) increase in frequency together, causing the relative number of females (black dashed lines) to decline. For designs 3 and 4, loss-of-function mutations at the X-shredder (α-XS, blue dashed-dotted lines) are selected for, replacing α **(A, C)**. Since α-XS is identical to α in designs 1 and 2, the construct continues to reduce the relative number of females. If the population is not eliminated, β is eventually replaced by the resistant b allele and α-XS is replaced by the wild-type A allele, allowing the population to recover. For design 5, loss-of-function mutations at the X-shredder (β-XS, red dashed-dotted lines) are also selected for, but increases in frequency more slowly than the α-XS allele in Designs 3 and 4, and the intact β construct persists longer. For all designs, loss-of-function mutations at each of the other components (Cas-9, gRNA$_A$ and gRNA$_B$) remain at low frequency, having negligible impact on the efficacy of the designs **(B, D, F)**.



S7 Fig. Loss-of-function mutation rates of 10e-4 have minimal impact on the extent of population suppression. Solid lines are for baseline conditions where constructs remain intact after release, while dashed lines are for homing-associated loss-of-function mutations occurring at each component of each construct with probability 10e-4.