TITLE:

The haplolethal gene *wupA* of Drosophila exhibits potential as a target for an X-poisoning gene drive.

Clancy D. Lawler, Natalia Hernandes, Ana Karla Parra Nuñez, Soumitra Bhide, Simon Baxter and Charles Robin*

School of BioSciences, The University of Melbourne, Victoria, Australia

***Corresponding author:**

Charles Robin
Building 184, School of BioSciences, Parkville Campus,
The University of Melbourne, Victoria 3010 Australia
crobin@unimelb.edu.au

Running head: A target for a haplolethal gene drive

Abstract

A synthetic gene drive that targets haplolethal genes on the X-chromosome can skew the sex ratio towards males. Like an 'X-shredder' strategy it does not involve 'homing' and that has advantages including the reduction in gene drive resistance allele formation. We examine this 'X-poisoning' strategy by targeting four of the 11 known X-linked haplolethal/haplosterile genes of *Drosophila melanogaster* with CRISPR/cas9. We find that targeting the *wupA* gene during spermatogenesis skews the sex ratio so fewer than 14% of progeny are daughters. That is unless we cross the mutagenic males to X^XY female flies that bear attached-X chromosomes, which reverses the inheritance of the poisoned X chromosome so that sons inherit it from their mother; in which case only 2% of the progeny are sons. These sex ratio biases suggests that most of the CRISPR/cas9 mutants we induced in the *wupA* gene are haplolethal but some are recessive lethal. We have used simulations to model release strategies of a gene drive where an X-poisoning Y chromosome is introduced to invasive populations of the pest *Drosophila suzukii*. While the reproductive output of carriers of X-poisoning drives is less than that of X-shredding drives due to their post-zygotic mode of action, the threshold dynamics of an X-poisoning drive result in more predictable outcomes than X-shredders that tend to exhibit 'chasing' more often. Like *D. melanogaster* and *D. suzukii*, the *wupA* orthologs in *Anopheles* mosquitos are found on X chromosomes making it a viable target in multiple species.

Introduction

For many decades it has been envisioned that insect populations that vector diseases of humans and animals or that are pests of food crops could be controlled using genetic methods (Serebrovsky 1969; Knipling 1955; Curtis 1968; Whitten 1985; Burt 2003). Indeed, Sterile Insect Techniques have successfully controlled fly species (Bushland, Lindquist, and Knipling 1955; Hendrichs, Franz, and Rendon 1995; Scott *et al.* 2017) and field trials relying on self-limiting transgenes are currently being deployed and assessed (Yao *et al.* 2022; Waltz 2021). Recently, the prospect of synthetic gene drives, a distinct class of population manipulation, where transgenes can be engineered to spread through otherwise natural populations of insect pests has received much attention (Alphey *et al.* 2020; Hay, Oberhofer, and Guo 2021; Champer, Buchman, and Akbari 2016). The effectiveness of various gene drive designs, many of which use CRISPR/cas9 machinery, have been assessed in laboratory populations (Chen *et al.* 2007; N. Windbichler *et al.* 2007; Akbari *et al.* 2014; Kyrou *et al.* 2018; Oberhofer, Ivy, and Hay 2019; Webster, Vella, and Scott 2020; Yang *et al.* 2022). Many of these require 'homing' where double-stranded DNA breaks must be repaired by homologous recombination (HR) so that the transgenic selfish elements are copied and thereby spread. A drawback of such homing designs has been that some double stranded breaks can be repaired using the non-homologous end-joining (NHEJ) pathway. In these instances not only does homing fail but mutations that are resistant to subsequent CRISPR/cas9 cleavages can be introduced into the population (Nikolai Windbichler *et al.* 2011; Gratz *et al.* 2014; Champer *et al.* 2017; 2018; Hammond *et al.* 2017).

One gene drive design that does not require homing is referred to as the X-shredder (Galizi *et al.* 2014). It targets highly repeated sequences on the X chromosome during spermatogenesis such that X-bearing sperm are inviable, and the drive skews the sex ratio towards males. If the X-shredding transgenes could be placed on the Y chromosome, then the pattern of fathers-only-having-sons will mean the sex ratio will become more biased over the generations and the population will approach extinction. Originally, the X-shredder was attempted in lab populations of *Anopheles*

gambiae mosquito using the endonuclease *PpoI*, but subsequent implementations in *A. gambiae* mosquitos, *Drosophila melanogaster and Ceratitis capitata* fruit flies have deployed CRISPR/cas9 machinery (Galizi *et al.* 2014; Fasulo *et al.* 2020; Meccariello *et al.* 2021).

A related approach, dubbed 'X-poisoning', also targets loci on the X chromosome; not so the chromosome is physically destroyed but so that mutations are introduced such that progeny receiving the X chromosome will not be viable (Fasulo *et al.* 2020). This strategy uses CRISPR/Cas9 to target X-linked haplolethal genes. Although haplolethal genes are typically thought of as genes where both alleles of a diploid organism need to be functional, X-linked haplolethal genes can occur in many dipterans because their dosage compensation mechanism elevates the X chromosome-encoded output in males (that is in contrast to eutherian mammals where there is silencing of X loci in females and therefore haplolethals on the X would be incompatible with maleness) (Rose *et al.* 2016; Cook *et al.* 2012). There may be a few potential advantages of X-poisoning over X-shredding. Firstly, X-poisoning does not rely on natural repeats occurring exclusively on the X chromosome and theoretically this may increase its applicability to some species. Secondly, CRISPR cutting in one or a few sites on the X chromosome might occur more readily than cutting the X chromosome at an overwhelming number of places, as is required for X-shredding. Thirdly, X-shredding has been shown to be ameliorated by the NHEJ pathway thus potentially limiting its utility (Fasulo *et al.* 2020). Fourthly, as we explore in this manuscript, it may be that post-zygotic effects of X-poisoning may change the threshold dynamics of gene drive invasion.

While informative, laboratory cage experiments can fail to incorporate population dynamics of natural populations. Therefore, computational simulations are used to estimate the impact of drift, migration, mutation, relative fitness, population structure and environmental stochasticity upon drive outcome. For example, seasonality (Eckhoff *et al.* 2017), release range (North, Burt, and Godfray 2013) and spatial structure have been shown to alter simulation outcomes. While simulations of panmictic populations predict a suppression drive will either fix, be lost, or reach a stable equilibrium (Burt 2003; Larner *et al.* 2019; Prowse *et al.* 2017), spatially explicit simulations show that a suppression gene drive in continuous space may undergo

cyclic patterns of local population elimination followed by recruitment of both wildtype and gene drive individuals through immigration (Champer *et al.* 2021; Paril and Phillips 2022). This may lead to unstable drive frequencies and cyclic subpopulation elimination and recolonisation that has been termed a gene drive 'chase' (Champer *et al.* 2021). This instability, and unpredictability, is likely to be an undesirable feature of a population control system as it may lead to ambiguity in drive frequencies and long-term persistence of the gene drive. Gene drives with strong threshold dependent dynamics may be less likely to undergo a population chase, as the required threshold to drive through the population is unlikely to be reached by migration alone (Buchman *et al.* 2018; Sánchez C. *et al.* 2020). How these dynamics are likely to play out in an expanding population is currently not well studied.

Here we describe the targeting of haplolethal genes on the X chromosome of *Drosophila melanogaster* with CRISPR/cas9. The loci differ from the two targeted in the X-poisoning study of Fasulo *et al.* (2020) as we examine three other ribosomal protein encoding genes and the haplolethal gene *Wings up A (wupA)*. In contrast to the ribosomal protein genes which might be required for the viability of most cell types including those in spermatogenesis, *wupA*, encodes a troponin that is expressed in muscles from mid embryogenesis. We therefore sought experimental affirmation that these genes are indeed haplolethal genes and whether they could be valuable targets for X-poisoning gene drives. We then simulate scenarios where X-poisoning constructs are hypothetically placed on the Y chromosome so that they drive through populations. As the postzygotic lethality of many haplolethal mutations is likely to reduce the output of fertile offspring for drive carrying males (Prado, Canal, and Ferrús 1999; Cook *et al.* 2012), we model the drive's dynamics in comparison to the pre-zygotic X-shredder drive. The simulations model a hypothetical incursion of *Drosophila suzukii*, an agricultural pest of global significance closely related to *D. melanogaster* (Mazzi *et al.* 2017; Ørsted and Ørsted 2019; Yeh *et al.* 2020; Knapp, Mazzi, and Finger 2021). Our spatially explicit models of an X-poisoning gene drive and an X-shredder gene drive were designed to answer three main questions. Firstly, how do these two population control methods function in a spatially explicit expanding population? Secondly, under what circumstances, if any, does an X-poisoning drive outperform an X-shredder drive? Thirdly, how do changes in release strategy impact each of the gene drives?

Materials and Methods

gRNA design and cloning

A combination of ChopChop v3 (Labun *et al.* 2019) and CRISPR Optimal Target Finder (Gratz *et al.* 2014) was used to identify the gRNA target sites with the highest efficiency scores with a low likelihood of off-target activity. All gRNA sequences were checked against the DGRP reference panel using FlyBase's JBrowse (Larkin *et al.* 2021) to avoid standing variation at the target site that may give natural resistance.

The design protocol of (Port and Bullock 2016) was used to clone gRNA sequences into the pCFD5_w plasmid (Addgene #112645). Three separate plasmids were generated that targeted haplolethal genes on the X chromosome; pRpS19a_RpL35, pRpL36_RpL35, and p*wupA*. pRpS19a_RpL35 encoded four gRNAs, two targeting *RpS19a* (*RpS19*_RNA1: GAAGGATATTGACCAGCACG, *RpS19*_RNA2: AACCGACTCCAGCGGGACTG) and two targeting *RpL35* (*RpL35*_gRNA1: GTGCTCCGAGCTGAGGATCA, *RpL35*_gRNA2: CACAATGTAGACGCGAGCGA). pRpL36_RpL35 encodes four gRNA, two targeting *RpL36* (*RpL36*_gRNA1: GCTGGCTATTGGCCTGAACA, *RpL36*_gRNA2: CATGCGCGACTTGGTCCGCG) and two targeting *RpL35* (as aforementioned). Each of the three sgRNA's used to target *wupA* (*wupA*_gRNA1: ACCAAAAACACAAATCAAAA, *wupA*_gRNA2: TGAGGTGCGCAAGCGCATGG, *wupA*_gRNA3: CGCATCATCGAAGAACGTTG) would affect all 13 alternate transcripts annotated in Flybase. The PAM site for *wupA*_gRNA1 corresponds to the most common start codon and is in the 5'UTR of transcripts using the alternate start codon, the PAM site for *wupA*_gRNA2 corresponds to the alternate start methionine which is a codon used by in all transcripts, and that for *wupA*_gRNA3 is in a coding exon common to all transcripts (Figure 1). All primers used and sgRNA sequences are tabulated in Table S1.

Microinjections

The pRpS19a+RpL35 and p*wupA* were microinjected into the 09w fly line while the pRpL36+RpL35 was injected into the 10w fly line. These two fly lines were obtained from Trent Perry, Bio21 Unimelb who had generated them using the Bloomington stocks #3605 (w[1118]) and #25709 (y[1] v[1] P{y[+t7.7]=nos-phiC31\int.NLS}X;

177 P{y[+t7.7]=CaryP} attP40) and #25710 (P{y[+t7.7]=nos-phiC31\int.NLS}X, y[1] sc[1]
178 v[1] sev[21]; P{y[+t7.7]=CaryP}attP2) to generate second chromosome landing site
179 flies (09W) and third chromosome landing site flies (10W) in a *white* null background.
180 Insertion events were validated by sequencing and single integration lines were
181 maintained.

182 **Assessing sex ratio biasing capabilities of sgRNA constructs**

183 To assess the sex ratio biasing rates of each of the sgRNA expressing
184 constructs, we first generated male flies that expressed both Cas9 and sgRNAs in their
185 germ cells. Individual females homozygous for each of the three sgRNA constructs
186 (RpS19a+RpL35, RpL36+RpL35 and *wupA*) were crossed to males containing Cas9
187 under the control of the *nanos* promoter (Either NIG CAS-0001; y^2 cho^2 v^1; attP40{nos-
188 Cas9}/CyO which carries a recessive lethal allele on the second chromosome and
189 cannot be made homozygous or a line we generated that has {nos-Cas9}attP40 placed
190 in the 09w second chromosome attP40 landing site(referred to herein as NH-
191 nosCAS9). The paternal inheritance of cas9 prevented maternal deposition and
192 somatic activity of the drive (Champer *et al.* 2019). Initially, single males heterozygous
193 for both cas9 and one of the sgRNA constructs were crossed to three 09W 3–4-day
194 old virgins and allowed to mate and lay in a vial for seven days before being cleared
195 and all offspring that emerged counted. Subsequent crosses included single nos-
196 cas9/Wup-sgRNA males left to mate with single females for four hours, or alternatively
197 crossed to three-day-old virgin females with the genotype C(1)DX,y^1,f^1/Y (Bloomington
198 Stock number 4559), which is an 'attached-X' line forces paternal X chromosome
199 inheritance.

200 **Simulations**

201 All models were generated using SLiM 3.6, an individual-based forward
202 population genetic simulation program (Haller and Messer 2019) and run on the
203 Spartan cluster at the University of Melbourne. The simulations were designed to
204 replicate the biology of *Drosophila suzukii* (Supplementary Table S2; Krüger *et al.*
205 2019; Lanouette *et al.* 2020) with some parameters taken from *Drosophila*
206 *melanogaster* data (Morrow, Stewart, and Rice 2005; Schnakenberg, Siegal, and
207 Bloch Qazi 2012), and with overlapping generations and an expanding population in a
208 two-dimensional space. Time was measured in generations with each generation

being the length of time taken for an embryo to reach reproductive maturity. To simulate the expansion of the invading population, wild-type individuals started within one generation's movement of the XY coordinates (0,0) within the square two-dimensional space (Video S1-S8). The maximum per generation movement within the simulated two-dimensional square varied as a test parameter with values between low, medium-low, medium-high, and high. The per generation movement varied around a normal distribution with standard deviation as a percentage of the entire simulated area – high per-generation movement results in approximately 95% of individuals moving ~0-6% of the entire simulated area and the low per-generation movement speed resulting in approximately 95% of individuals moving between ~0-4% of the entire simulated area per-generation along either axis with medium-low and medium-high sitting equidistant between these values. Both the dynamics of a X-poisoning drive and a X-shredder drive were tested within the context of these simulations.

Four release strategies, each occurring at least 10 generations after the population started its invasion into the space, were implemented for each of the two gene drive systems:

1. The total number of gene drive individuals were released once uniformly over the entire occupied area.
2. The total number of gene drive individuals were released only at the point where the invasive population originated.
3. A proportion of the total number of gene drive individuals were released uniformly over the entire inhabited area every multiple of the release generation.
4. A proportion of the total number of gene drive individuals were released only at the point where the invasive population originated every multiple of the release generation.

The number of released individuals varied between 100 and 993. The generation of the gene drive release varied between generation 10 and 30 with the multiple release simulations occurring at an interval of between 1 and 20 generation between generation 10 and generation 110 – all simulations have a 10 generation burn in period with generations counted after this initial period such that release generation measures the number of generations after the burn in period. Both the multiple interval-based release and the single release strategies maintain the same total number of released individuals – either the total release number is released in a single go, or a

242 proportion of the total release number is released every generation interval. The X-
243 shredder gene drive was encoded so that the sex ratio of the offspring was biased
244 towards males whereas the X-poisoning drive resulted in females (born to a drive
245 containing father) being made non-viable before they reached sexual maturity –
246 resulting in post zygotic lethality. The proportion of 100 replicate simulations that led to
247 population eradication within 110 generations was assessed. For further information
248 on simulation variables see Supplementary Table S2 and for the simulation code see
249 files S1 and S2.

250 Statistical Analyses

251

252 All statistical analyses were undertaken in R (R Core team 2023). Sex ratio biasing
253 in the *Drosophila* X-poisoning gene drive crosses were analysed using an unpaired
254 two-tail t-test with a Bonferroni correction for multiple comparisons where applicable.
255 Simulation data was generated using Spartan cluster at the University of Melbourne.
256 Each combination of variables was independently run 100 times and the average
257 outcome shown in a heatmap. The R code used to analyse the data is available in
258 file S3.

259 Results

260

261 Four of the haplolethal genes described on the X chromosome of *Drosophila*
262 *melanogaste*r (Cook *et al.* 2012) were targeted using three multi-sgRNA constructs
263 and cas9 expressed in the male germline via a *nanos* promoter (Figure 1). The
264 expectation was that males carrying the cas9 and sgRNA transgenes would have a
265 reduced number of viable daughters relative to controls yet the same number of sons
266 (Figure 2a). All the crosses targeting ribosomal protein encoding genes
267 (RpS19a+RpL35, RpL36+RpL35) yielded significantly fewer offspring when compared
268 to the controls ($p<0.0001$, t-test with Bonferroni correction; Figure 2b). The crosses
269 involving the RpS19a+RpL35 sgRNA construct followed expectations with significantly
270 fewer daughters than sons ($p<0.0001$, t-test with Bonferroni correction) and no
271 significant difference in the number of sons compared to the control. In contrast the
272 RpL36+RpL35 crosses yielded significantly fewer sons *and* daughters compared to

control ($p<0.05$ and $p<0.01$ respectively, t-test with Bonferroni correction) without a significant sex ratio bias.

When the CRISPR machinery was targeted to *wupA* significant skews in sex ratio towards maleness was observed ($p<0.0001$, t-test with Bonferroni correction, Fig. 2). However, we initially found the expression of sgRNAs targeting *wupA* did not result in a significant reduction in the total number of offspring produced when compared to controls (Fig. 2b). The highly significant reduction in the number of female offspring ($p<0.0001$, t-test with Bonferroni correction), was offset by the significantly higher number of male offspring compared to control ($p<0.0001$, t-test with Bonferroni correction). We hypothesised that the lack of reduction in reproductive output may be due to a carrying capacity within the vials with an excess of embryos being laid during the seven day period where remating was allowed. To address this possibility, single males heterozygous for both X-poisoning drive components (ie. the *wupA* targetting sgRNAs and nos-cas9) were placed in vials with single females for four hours only. In these single pair crosses, a significant reduction in the total number of offspring ($p<0.01$, t-test with Bonferroni correction) as well as a highly significant change in sex ratio was observed ($p<0.0001$, t-test with Bonferroni correction; Figure 2c) and there was no significant difference in the number of males produced when compared to the control.

To understand why the sex biasing was not absolute, X-chromosomes that had survived being passed through the paternal germ line were characterized. If the targeted loci had been cut and reannealed, indels or single nucleotide mutations may have been introduced, and they would be revealed by sequence analysis at the sites targeted by the sgRNA's. To simplify the sequence analysis, we used X^XY females which reverse the inheritance of the paternal X's so that they are passed to sons. Consistent with expectations, males expressing *nanos*-Cas9 and sgRNA's targeting *wupA* sired significantly more daughters than sons (the sex ratio bias being flipped due to sons paternally inheriting the X chromosome; $p<0.001$, t-test with Bonferroni correction). The RpS19a+RpL35 cross showed no significant sex ratio bias but had significantly fewer male offspring than the control and no significant difference in the number of females (Figure 2d; $p<0.0001$, t-test with Bonferroni correction). There was no reduction in the number of offspring produced by males expressing gRNA targeting RpL36+RpL35.

306 The X chromosomes of a total of 60 surviving sons (14 for *wupA*, 22 for
307 RpS19a+RpL35 and 24 for RpL36+RpL35) from 15 independent crosses were
308 sequenced and only the offspring of one male showed any sign of CRISPR cutting. A
309 complex indel resulting in a net 9 nucleotide insertion that did not introduce a premature
310 stop codon was recovered from a cross involving the RpL36+RpL35 sgRNAs (Figure
311 S1). The fact that this individual survived suggests that gene drive resistance alleles
312 could arise against RpL35 gRNA1. The data also concurs with the idea that these
313 genes are indeed lethal genes as no frameshifting indels or nonsense mutations were
314 recovered. More generally, these data are consistent with the notion that most of the
315 surviving progeny carrying a paternal X never had their X chromosome cut by the
316 CRISPR machinery.

317 The above results suggest that *wupA* could be a good target for an X-poisoning gene
318 drive. To create such a drive, transgenes encoding cas9 and the sgRNA's targeting
319 *wupA* would ideally be placed on the Y chromosome. Placing transgenes on the Y
320 chromosome has been technically challenging although it has been done (Gamez *et*
321 *al.* 2021). Indeed, we examined the one *D. melanogaster* line currently available that
322 has a cas9 gene on the Y chromosome (BL91386). The cas9 of this line uses a *vasa*
323 promoter and this expresses outside the germline as well as in it. A cross between
324 males with this vasa-cas9 Y chromosome with females bearing *wupA* targeting
325 sgRNA's yielded almost entirely daughters (264 females and two males that were small
326 and infertile). Presumably the males that would have cas9 and sgRNA expression in
327 their germline die before they reach adulthood because somatic expression of the
328 vasa-cas9 with the ubiquitously expressed *wupA* sgRNA's is lethal.

329 However, as an X-poisoning Y chromosome could in theory be built with a cas9
330 restricted to the germline, and as the *wupA* gene is found on the X chromosome of
331 other Drosophila species including the pest species *Drosophila suzukii*, we set out to
332 model an X-poisoning strategy deployed in a hypothetical invasion of *D. suzukii*.

333 **Simulations of gene drives in an expanding population**

334 *Drosophila suzukii* is an invasive species that has recently spread throughout
335 the United States and Europe and it has the potential to spread in Australia if an
336 incursion establishes (Maino, Schouten, and Umina 2021). We therefore developed
337 spatially explicit models (in SLiM; Haller and Messer 2019) to explore the dynamics of

338 an X-poisoning drive and an X-shredder drive in expanding populations. The models
339 incorporated parameters that attempted to capture key aspects of *D. suzukii* biology
340 such as seasonal fluctuations in population size (Stockton, Wallingford, and Loeb
341 2018). The simulated space was a two-dimensional square with the invasive
342 population expanding from the bottom left corner (videos S1-S8). Populations differing
343 in movement rates were simulated and gene drive flies were released a varying
344 number of generations after the incursion and at various release sizes (Figure 3). We
345 contrasted models that used X-poisoning, in which daughters arising from gene drive
346 males did not reach reproductive maturity, to X-shredding where fathers only produced
347 Y-bearing sperm. As the former is post-zygotic and the former pre-zygotic, X-poisoning
348 drives had half the offspring of X-shredding drives.

349 We first modelled the scenario where gene drive individuals were released only
350 once at the incursion point. The X-poisoning drive failed to eradicate the population
351 under all tested parameters while the X-shredder drive showed increased eradication
352 likelihood with increased movement of individuals (Figure 3a). In these simulations the
353 X-shredder is largely unimpacted by 'release size' and 'release generation', although
354 a mild seasonality effect is observed in the later (see below). As the degree of
355 movement affects the distance from which a mate can be selected, it impacts geneflow
356 and metapopulation dynamics; low movement of individuals results in small
357 populations with uninhabited areas between them. If suppression occurs more quickly
358 than gene flow between the subpopulations, the X-shredder is unable to suppress the
359 entire metapopulation. As a result, the likelihood of eradication given a single release
360 at the incursion point is largely dependent on the degree of movement and the resulting
361 population geneflow (Figure 3a).

362 Next, we modelled multiple releases of gene drive individuals at the incursion
363 point. That strategy slightly increases the likelihood of eradication for the X-shredder
364 while maintaining the relationship between eradication likelihood and movement
365 (Figure 3b). Note that the same number of gene drives individuals were released as in
366 the 'single release', it was just that they were distributed over multiple releases.

367 We then simulated the release of gene drive individuals across the range of the
368 invasive species rather than just at the point of origin and found that to be a much more
369 effective for both drive types in both single and multiple releases (Figures 3c and 3d).

The simulated winter event impacts the likely outcome for both gene drives in these release strategies, appearing as a striated change in eradication likelihood at release generation 1, 10 and 20. In the simulated winters that occur every ten generations, no offspring are produced, and spatial competition is increased by 60%. Releasing the X-poisoning drive individuals uniformly over the invaded area immediately after incursion identification (immediately after the ten generation burn in period), or timing it with the fluctuations in population size, due to the effects, of winter gives the greatest likelihood of eradication (Figure 3c and d). The X-shredder drive shows similar dynamics during low movement speeds but is less impacted by release time at high movement speeds for the multiple release strategy.

X-poisoning appears to be as good as X-shredding when releases occur across the range and the movement of individuals is low and releases are well timed relative to the population declines in winter (Figure 3c and 3d). The inability of the X-poisoning drive to function at high movement speeds, or when released only at the incursion point, is indicative of a threshold dynamic within this drive. Unlike the X-shredder drive, the X-poisoning drive is unable to reach the threshold required to drive through the population by migration alone resulting in the threshold release rates required for eradication. The X-shredder is seen to have no threshold and the high degree of gene flow in the simulations with high movement enables the drive to eradicate the population more effectively.

Empirically informed sex ratio biasing:

In the above simulations the efficiency of the gene drives were maximized; the only fertile progeny of drive-bearing fathers, are sons. Next, we altered the simulations to incorporate more realistic gene drive efficiencies. These used the likelihood of female survival seen in the single mate crosses described in the X-poisoning crosses above (i.e. proportion of surviving females = 0.13), or the X-shredder drive sex ratio biasing rates observed by Meccariello *et al.* (2021) in *Ceratitis capitata* X-shredder (biasing offspring sex ratio towards male by 80%). In general, such changes made X-shredding as ineffective as X-poisoning given medium-high to high movement speed – the X-shredder drive still outperforms the X-poisoning drive at low movement speeds. However little effect was seen in single uniform release scenarios (compare Figure 3c to Figure 4a).

Gene drive chases:

For many of the simulations, especially for X-shredder gene drive models, populations were often observed to undergo cyclic fluctuations in population size and gene drive proportion. This was due to local eradication events, followed by recolonisation and subsequent reintroduction of the drive through migration – a gene drive chase. To identify the frequency of a chase occurring, the simulated space was split into 100 equal cells within the two-dimensional space. A chase was deemed to occur in a simulation if any cell went twice through the cycle of colonization and loss. As multiple releases could result in the misclassification, due to drive individuals being released into a cell rather than migrating into it, only single release strategies were examined. These simulations revealed that the likelihood of the X-shredder drive undergoing a gene drive chase was largely independent of release size, with release generation only having a minimal impact around winter (Figure 4b). As with eradication likelihood, the likelihood of gene drive chase for the X-shredder drive is most strongly impacted by the movement speed. The occurrence of a chase in a X-poisoning gene drive is rare, only occurring at low frequency under uniform release conditions (Figure 4b).

Gene drive persistence

In the case of a pest such as *Drosophila suzukii* the unpredictability of a chase dynamic is undesirable. As the X-poisoning gene drive has a lower occurrence of gene drive chase than the X-shredder under all release strategies and tested variables, we further examined whether X-poisoning provided an advantage over X-shredding by examining the persistence of gene drives within populations.

We assessed the number of simulated populations that had gene drives persisting in their last generation. In the simulations of X-poisoning drives there were none under any release strategy. Whereas the X-shredding simulations exhibited substantial rates of persistence particularly in scenarios where individuals within the populations had low to low-medium movement rates (Figure 4c). Due to the threshold dynamics of the X-poisoning drive, even if it were to undergo a chase it is unlikely to eradicate a population due to the migration of gene drive individuals alone. Unlike the X-shredder, which spreads quickly into an expanding population, the X-poisoning drive expands more slowly than the expanding wild-type population due to the reduction in

434 reproductive output from the postzygotic lethality of this drive. The reduction in
435 reproductive output acts as a fitness cost, resulting in the removal of the gene drive if
436 it is below the threshold required to drive. As a result, the X-poisoning drive either
437 eradicates the population or is removed from the population before the end of the
438 observed period.

439 Discussion

440

441 For an X-poisoning strategy to elicit an effect in a single generation the targeted
442 genes need to be haplo-insufficient. Such is the knowledge associated with
443 *Drosophila melanogaster* that it is known there are eleven loci on the X chromosome
444 of *D. melanogaster* that are haplolethal or haplosterile (Cook *et al.* 2012).
445 Haplolethal genes can only exist on the X chromosome in species where dosage
446 compensation between the homogametic and heterogametic sex is due to
447 upregulation - doubling the output of the X chromosome in males, rather than the
448 inactivation of the X as seen in humans.

449

450 Eight of the X-linked haplolethals of *D. melanogaster* encode ribosomal proteins,
451 which contribute to the translational machinery of cells, and an insufficient quantity of
452 them leads to lethality or to the Minute syndrome where flies are tiny and sterile
453 (Marygold *et al.* 2007). Previously, Fasulo *et al.* 2020 pioneered the 'X-poisoning'
454 strategy by targeting Ribosomal protein genes S6 and S5a. They found that when
455 one particular sgRNA targeting RpS6 was combined with cas9 driven by a sperm-
456 specific beta tubulin 85D promoter the sex ratio was biased so that greater than 92%
457 of progeny were males. Our targeting of RpL36, RpL35 and RpL19a did not produce
458 such extreme biasing. However, Fasulo *et al.* (2020) also showed heterogeneity in
459 the success of their sgRNA's with different guide RNA's targeting the same genes
460 skewing to various extents. Therefore, the failure to see skewing in our combination
461 of RpL36 and RpL35 sgRNA's or the skew to only 80% males in the RpL35 and
462 RpL19a combination, may well have more to do with the particularities of guide RNA
463 efficacy than being a property of the genes they target.

464

465 The gene that showed the greatest skewing in our X-poisoning experiments was the
466 only non-ribosomal protein haplolethal gene that has been molecularly characterised

on the X chromosome. The Wings-up-A (*wupA*) locus of Drosophila encodes Troponin I, a protein that inhibits the interaction of myosin and actin in muscles. It is also thought to have non-muscle functions that relate to the nuclear localization of at least one of its nine isoforms (Casas-Tintó and Ferrús 2021, Flybase). The gene has 12 exons that are variously combined to produce 13 alternate transcriptional splice forms. Two of the three sgRNAs used in our study target exons common to all splice forms, and the third targets a PAM site that is found in the first two codons of isoforms with the most common start methionine. The latter site was previously targeted with CRISPR by Casas-Tintó and Ferrús (2021) and the resulting haplolethality is consistent with our findings.

Curiously, we found that the skew in sex ratio was more extreme when the poisoned X chromosome was passed to sons (via attached-X females) rather than to daughters (figure 2d versus figures 2b and 2c). As the fathers in these crosses are the same, this difference is not due to the generation of X-poisoned alleles. Instead, heterozygosity in daughters may protect more than the exposed hemizygosity of sons, which suggests that some of the CRISPR generated alleles may encode recessive lethals. An alternate explanation is that there is something else about maleness that makes the CRISPR *wupA* alleles more potent in males than females.

A surprising finding of Fasulo *et al.* (2020) was that some alleles among the survivors contained frameshift mutations. This led the authors to suggest that the targeted ribosomal protein genes may not be haplolethal genes after all and that previously studied alleles may have harboured dominant negative mutations instead. In our analyses, we did not find such frameshifting alleles among individuals carrying X-chromosomes that had passed through X-poisoning males. Instead, except for a single in-frame alteration, the loci showed no signs of being cut at all. While our analyses did not survey as many individuals as Fasulo *et al.'s* study, it perhaps avoided issues of sequencing errors, copy number variation or chimerism, which provide alternate explanations for Fasulo *et al.*'s (2020) viable frameshifts in pooled ribosomal-protein gene sequences.

WupA has very low expression in the early stages of embryos (first 8 hours) which contrasts to many ribosomal proteins because it is probably not required for the

viability of all cells. Perhaps this feature explains why the number of sons is not reduced in the *wupA* cross, whereas they are reduced in the ribosomal-protein crosses. Such an effect may also extend back to the fitness of Y bearing sperm produced by male carriers of the *wupA*-poison. They may be as viable as wildtype sperm in a way that is not true for Rp-poisoning. This makes it an attractive target for future X-poisoning strategies.

WupA is a highly conserved gene that is found on the X chromosome on numerous dipteran species, including some important pests. *Drosophila suzukii,* an invasive pest of soft-skinned fruits, appears to share all 12 exons with *D. melanogaster* and all the predicted proteins have greater than 98% amino acid identity with *D. melanogaster* homologs. In fact, two of the three sgRNA's used here in *D. melanogaster* match *D. suzukii* targets perfectly, and the third has only one nucleotide different. *WupA* of Anopheles mosquito's has a slightly different gene structure (eg. Exon 1 and Exon 2 are fused eg. XM_053811545) however it is also found on the X chromosome in these species suggesting it could be a target of X-poisoning strategies in the many pest species of this genus.

Our modelling compared X-poisoning to X-shredding drives and suggested that the latter is often more effective at eradication in an expanding population. The X-shredder gene drive can expand into the expanding population of insects, eradicating them as it goes. It does this more effectively than X-poisoning because its bearers were modelled as having twice the reproductive output when compared to the X-poisoning (this may be over-conservative as multiple mating's may introduce a frequency dependent effect that would be less harsh on the X-poisoning). As the expansion of the wild-type population is constrained by the ability to find a mate and produce offspring at the invasion front, the X-shredder drive can expand into the wild-type population at a slightly faster rate than the wild-type population can expand into the uninhabited space. This results in the expansion of the drive following the expansion of the population like a wave (e.g. Video S1).

The simulations also emphasize how important the extent of movement of individuals is to gene drive success. All interactions between individuals are impacted by movement: competition is based on the number of individuals within a generation's

movement, mating occurs only between individuals within interacting distance. As movement increases, the interaction distance increases, and gene flow within the population increases. Individuals are also able to colonise new uninhabited areas more quickly resulting in the space reaching carrying capacity more quickly. For X-shredding the greater the movement the more successful the gene drive. However for X-shredding the effect the effect is quite different; with eradication only successful when the movement of individuals is low. As the population size increases faster with greater movement of individuals, yet the number of released individuals is constrained (it is set to a certain size in our simulations, or to what is logistically possible in a real scenario), the proportion of released gene drive individuals within the population decreases and so in the high movement scenarios the released individuals never cross the necessary threshold required for population eradication.

While the likelihood of eradication would be a primary consideration of anyone deploying a suppression gene drive, many of our simulations showed that the X-shredder gene drive persisted until the end of the observed period. The persistence of the gene drive was particularly true when gene drive chasing occurred. Due to the drive suppressing the population, the X-shredder drive leaves uninhabited space behind it. If wild-type individuals move behind the expanding wave of the X-shredder individuals, they are then able to expand in the other direction and thereby escape the gene drive. This often results in a gene drive chase back in the other direction. Persistence of gene drives is an undesirable outcome for gene drive population control as it increases the length of time the drive is present within the population and that increases the likelihood that the drive will escape beyond the target area, potentially impacting the species in its native range. X-poisoning on the other hand has more of a threshold dynamic which limits the chasing dynamic. Thus, there are circumstances where an X-poisoning may be a better strategy than X-shredding.

Our models also illustrate several other features that would need to be considered before gene drive release. Features such as seasonal declines in population size had a noticeable effect. Perhaps most significantly our models demonstrated the importance of gene drive release strategies. They found it was much better to release the gene drive throughout the invasive species range rather than limiting the gene drive release to the incursion origin.

Data Availability Statement

All data is here or in the supplementary material.

Acknowledgements

We'd like to thank Owain Edwards and Max Scott for conversations that helped develop this research and Jackson Champer for suggestions about construct design.

Funding

The research was funded by an Australian Research Council Discovery Project Grant (DP190102512).

Conflict of Interests

We have no conflicts of interest to declare.

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Figure Legends

Figure 1: Targeting Haplolethal genes on the X chromosome. (a) Premeiotic cells expressing CRISPR/Cas9 (from the autosomes) that effectively target haplolethal loci on the X-chromosome (orange to red) will result in male only offspring. If the machinery were put on the Y-chromosome then inheritance of the elements will be limited to the male germ line and a gene drive would be created. (b) Eleven haplolethal/haplosteriles were reported by Cook *et al.* (2012), two (RpS6 and RpS5a) were targeted by Fasulo *et al.* 2020 and the location of the four loci targeted in this manuscript, and the number of sgRNA used (rounded blue rectangles) are shown. (c) The WupA gene encodes for thirteen transcript isoforms. SgRNA1-3 target three conserved regions of the gene's CDS.

Figure 2: Sex biases with X-poisoning. (a) A skew against daughters (red) is expected when sgRNA's target haplolethal loci on the X chromosome during gametogenesis. The number of sons is expected to remain unchanged as they would arise from sperm carrying the Y chromosome. (b) The observed amount of sex biasing skew ranged from nothing (RpL36+RpL35), to modest (RpL19a+RpL35), to promising (WupA). Note that the total number of offspring was also variously affected. (c) By excluding multiple mating the total number of offspring in the WupA cross was reduced and there was a strong skew towards males (d) CRISPR/cas9 fathers crossed to Attached X mothers, reverse the inheritance of the sex chromosomes so that sons inherited the CRISPR modified X chromosome, and daughters inherited the Y chromosome. (* $P < 0.05$, ** $P < 0.01$, ** $P < 0.001$ **** $P < 0.0001$, unpaired two-tail t-test with a Bonferroni correction for multiple comparisons).

Figure 3: Simulations of 'X-poisoning' and 'X-shredding' in expanding populations. The eradication probability of the invasive population is shown in a black to yellow scale with each coloured element representing 100 simulations. Each square represents simulations that varied by the number of gene drive individuals that were released and when they were released relative to the incursion of the

invading species. Four values of movement of individuals are shown (low movement, medium-low, medium-high, high). Simulations represented in (a) had the gene drive individuals released at a single time at the incursion point of the invasive species. Simulations represented in (b) involved multiple smaller releases at the incursion point. In the simulations of (c) and (d) the gene drive individuals were released across the occupancy range of the invasive species. Each data point represents the average outcome of 100 independent simulation runs.

Figure 4: Simulations with realized biasing efficiencies. In contrast to the analysis of Figure 3 which assumed the poisoning and shredding happened with absolute efficiency these simulations used the data generated here and in the paper of Meccariello *et al.* 2021 to inform sex ratio biasing rates. All the simulations shown here featured a single gene drive release across the present range of the invasive species. In the simulations of (a) the probability of population eradication is shown and seasonality can be seen as horizontal striations. In (b) the colour represents the likelihood that a gene drive ‘chase’ occur and in (c) the colour represents the presence of the gene drives at the end of the modelling. Each data point represents the average outcome of 100 independent simulation runs.

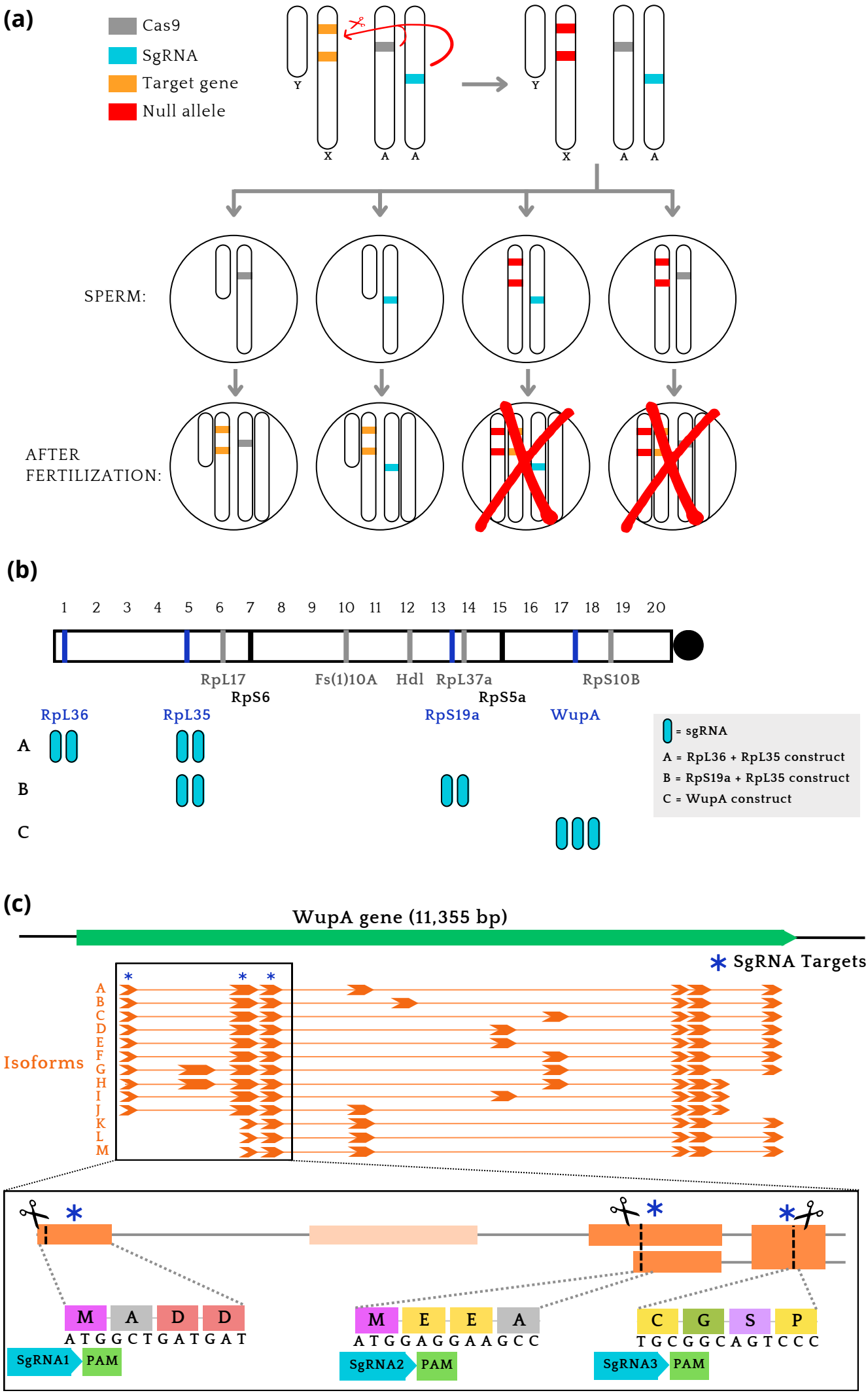
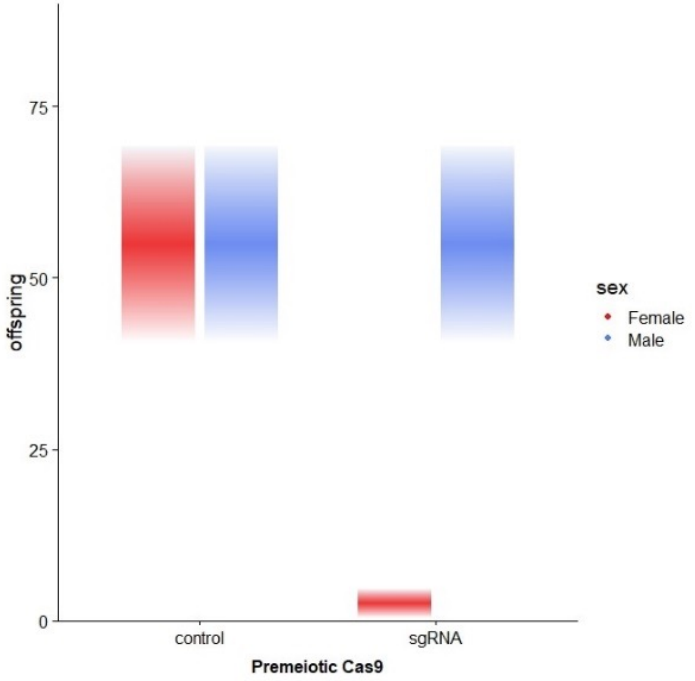
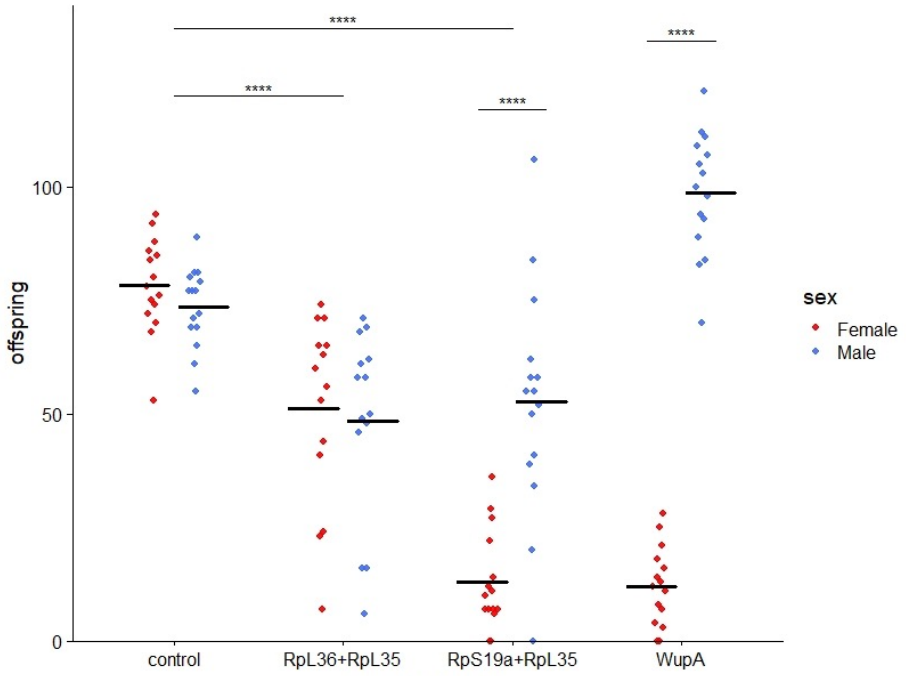

(a)
Cas9
SgRNA
Target gene
Null allele
Y
X
A
A
SPERM:
AFTER FERTILIZATION:
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
RpL17
RpS6
Fs(1)10A
Hdl
RpL37a
RpS5a
RpS10B
RpL36
RpL35
RpS19a
WupA
A
B
C
= sgRNA
A = RpL36 + RpL35 construct
B = RpS19a + RpL35 construct
C = WupA construct
(c)
WupA gene (11,355 bp)
SgRNA Targets
Isoforms
A B C D E F G H I J K L M
M A D D
A T G G C T G A T G A T
SgRNA1
PAM
M E E A
A T G G A G G A A G C C
SgRNA2
PAM
C G S P
T G C G G C A G T C C C
SgRNA3
PAM

Figure 2

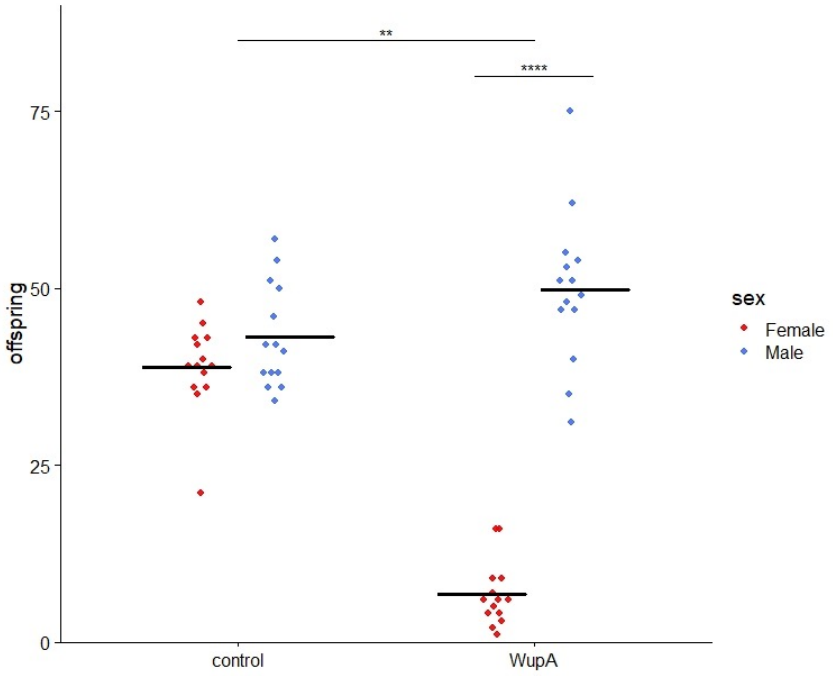
(a)



(b)



(c)



(d)

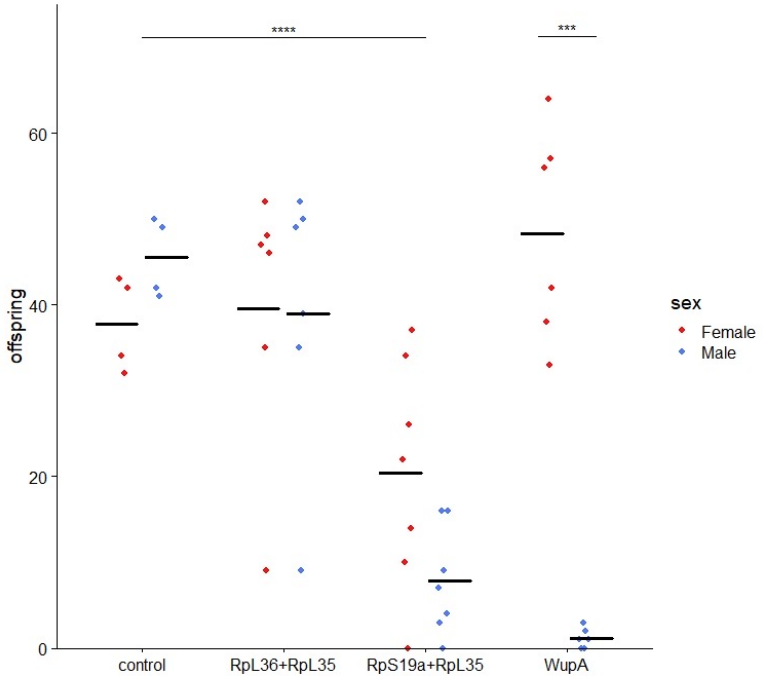


Figure 3

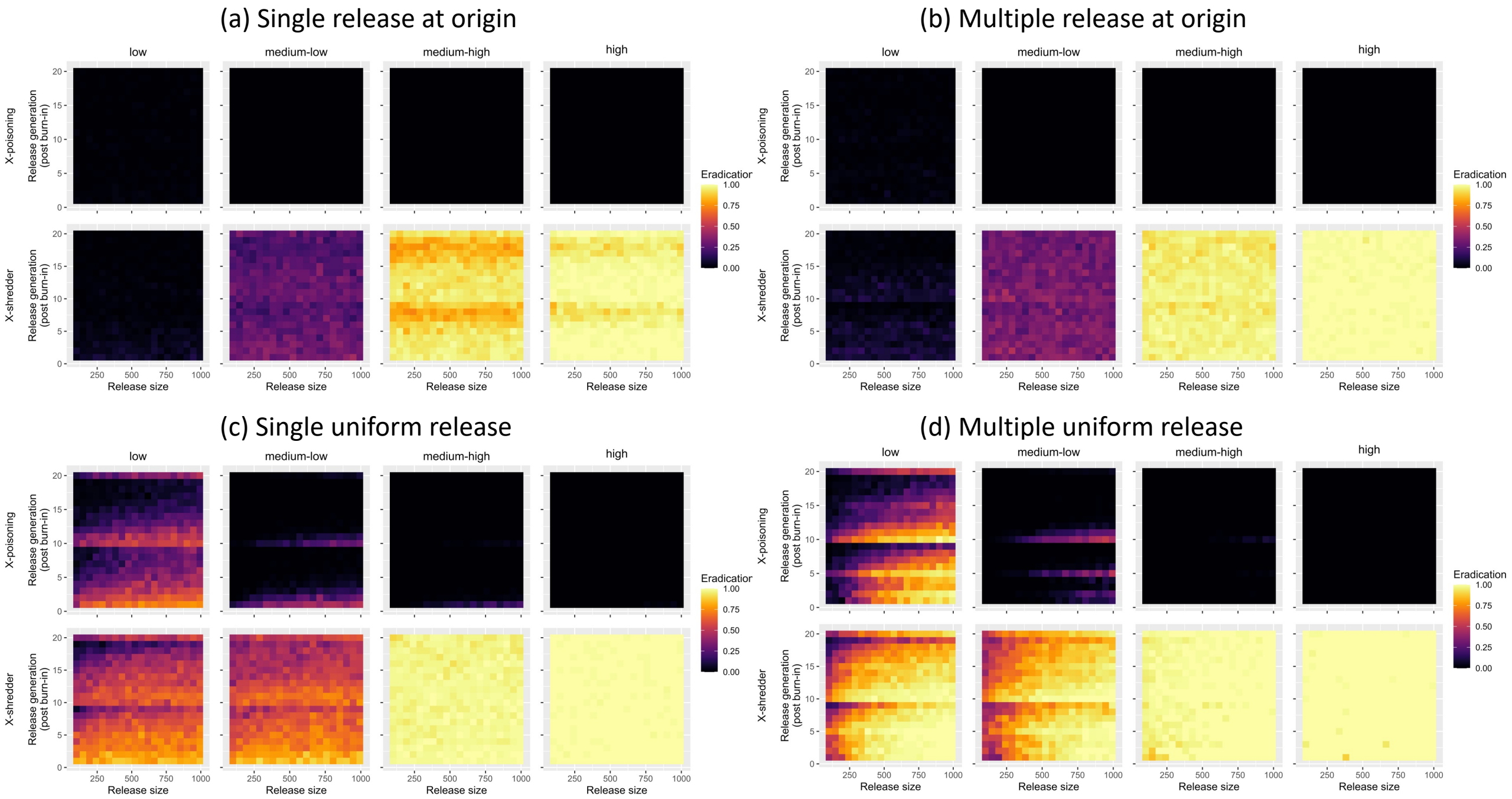
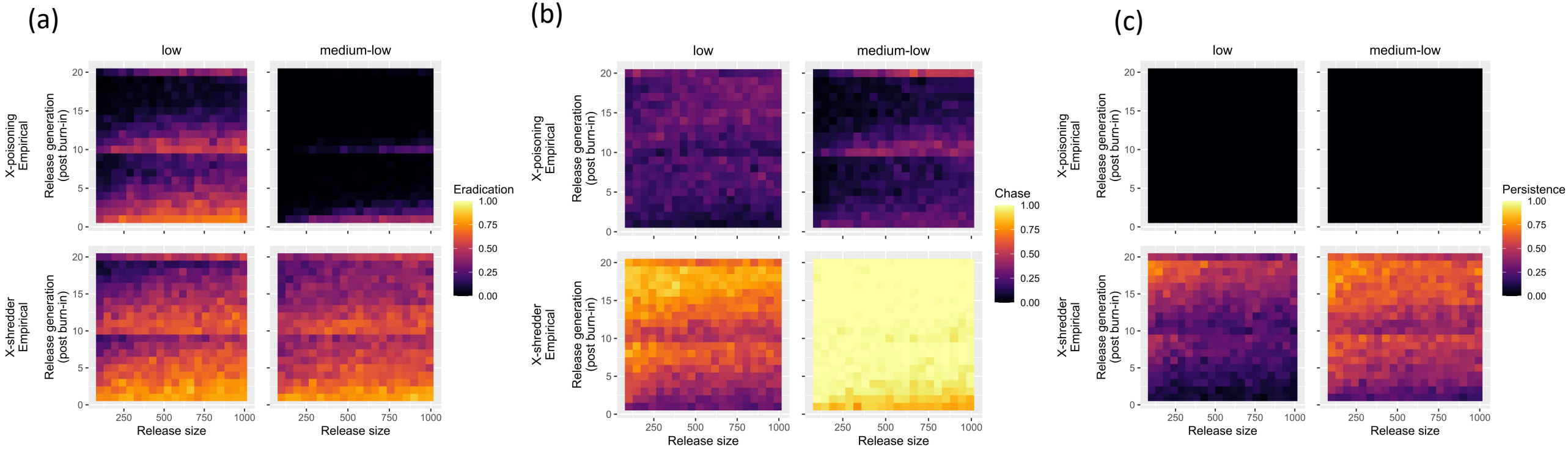

(a) Single release at origin
(b) Multiple release at origin
(c) Single uniform release
(d) Multiple uniform release
low
medium-low
medium-high
high
X-poisoning
X-shredder
Release generation (post burn-in)
Release size
Eradication
1.00
0.75
0.50
0.25
0.00

Figure 4


(a)
low
medium-low
X-poisoning Empirical
X-shredder Empirical
Release generation (post burn-in)
Release size
Eradication
1.00
0.75
0.50
0.25
0.00
(b)
Chase
(c)
Persistence