Towards Responsive Eco-technology: The Development of a Male Sex-biased Mouse

by

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B.S., University of Chicago (2016)

Submitted to the Program in Media Arts and Sciences, School of Architecture and Planning
in partial fulfillment of the requirements for the degree of

Master of Science in Media Arts and Sciences

at the

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Abstract

CRISPR-Cas systems have catalyzed the emergence of several synthetic population management strategies, like gene drives, for controlling pests and disease vectors. As these technologies garner greater visibility in both general and regulatory audiences, questions have arisen about the invasiveness of drive strategies and have underscored a need for community guidance in designing population management technologies. In heterogametic species, an engineered male-determining chromosome can serve as a method for providing robust and localized population suppression without the need for a gene drive. In mice, X-chromosome inactivation is mediated by X-inactive specific transcript (Xist) long non-coding RNA. I propose to encode a system on the Y chromosome to knock out a necessary region for proper X-inactivation in females. Loss of Xist gene function has no known effects in males or females with a dysfunctional maternal copy, however, females who inherit a dysfunctional paternal copy die at embryonic day 8.5. Thus, this results in a male sex-biased mouse. To create a daughterless mouse, my proof-of-principle design will include a constitutively expressed Cas protein with at minimum a two-guide array. Additionally, I will draw on the ecological species concept found in some cultures, like the Māori of New Zealand, to create an alternate eco-cisgenic version using cisgenic murine elements and a CRISPR system found in a commensal species of bacteria. Creating a cisgenic non-driving mammalian model of a genetic population suppression system would be a first-of-its-kind example to show how biological engineering design decisions can be congruent with culturally specific notions of ecology.

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Chapter 1

Introduction

Non-native invasive species present both ecological and economic threats to the communities where they are found. Invasive species are the foremost driver of extinction of birds and the second most driver of extinction for mammals and fish, globally (Clavero and García-Berthou 2005). On islands, a growing body of literature suggests that they are the greatest driver of population decline and species extinction, surpassing habitat destruction (Reaser et al. 2007; Chapin et al. 1998; Veitch and Clout 2002). In the United States, rodents like the Black rat (*Rattus rattus*), the Norway rat (*Rattus norvegicus*), the house mouse (*Mus musculus*) are responsible for at least 19 billion dollars in direct damage through food consumption and spoilage while also contributing to spreading diseases like plague, salmonellosis, and leptospirosis (Pimentel et al. 2000).

Methods for controlling invasive species have come to rely largely on anticoagulant poisons which cause unnecessary animal suffering and often result in the poisoning of children and wildlife (Parsons et al. 1996). A class of anticoagulant poisons known as "super-warfrins" are persistent and ubiquitous to the extent that secondary exposure to predators is also widely reported in addition to non-target rodent and bird species directly feeding on the baits (van den Brink et al. 2018). While anticoagulant poisons have been responsible for 85% of the market share of rodenticides, there is growing interest in the possibility of using genetic approaches to mitigate the harm done by poisons (Campbell et al. 2015; Esvelt et al. 2014).

1.1 Related Work

The most common genetic approach to pest management is the release of sterile males, typically derived through irradiation, transgenics, or cytoplasmic incompatibility. (Alphey et al. 2010). Sterile male release programs typically require large release sizes, on the order of ten-times the target population, over multiple generations to be effective (Burt and Deredec 2018). Such programs can be resource-intensive with respect to both labor and cost. Genetic approaches involving selfish elements that exhibit preferential inheritance, like gene drive, require radically fewer field inoculation sizes, on the order of one percent of the target population, yet carry the latent risk of being highly invasive (Noble et al. 2018).

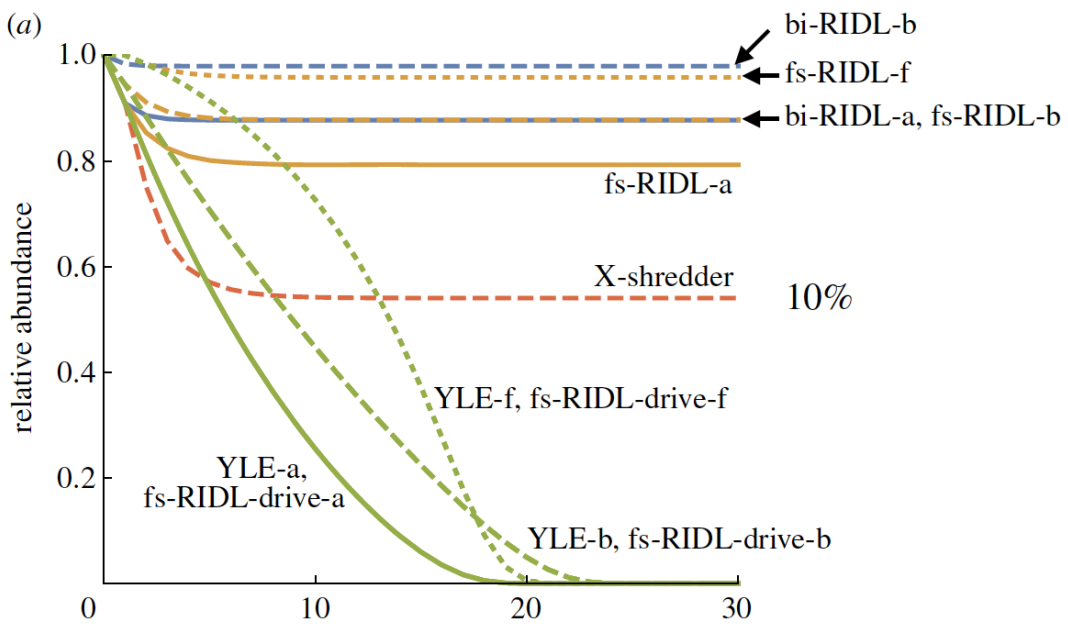

Figure 1-1: The time course of population control with different self-limiting constructs. Given the introduction of various constructs at 10% of initial male population in each subsequent generation, Y-linked editors that cause death after density dependance (YLE-a) provide population suppression comparable to constructs disrupting a dominant autosomal female-specific lethal gene that drives in males (fs-RIDL-drive-a), and is more effective than other approaches Burt and Deredec (2018).

Genetic approaches to pest management have seen particular success in the case of insects where two dominant modalities of thought have emerged, the use of bac-

terial symbionts and the creation of self-limiting transgenic organisms. The canonical use-case of bacterial symbionts in pest management is the use of *Wolbachia* in mosquitos. *Wolbachia* is a maternally inherited symbiont bacterium found in an estimated 60% of all insect species (Hilgenboecker et al. 2008). Beginning with the discovery that the wMelPop strain of *Wolbachia* was able to shorten the lifespan of some insects, and later that it is also able to reduce the ability of vector-borne viruses like Dengue, Chikungunya, and Zika to replicate and transmit in *Aedes aegypti*, The World Mosquito Program, a global non-profit organization, (formerly Eliminate Dengue) has run several pilot field deployments (Ritchie et al. 2015; Yeap et al. 2014). The initial field trials have been successful in Northern Queensland, Australia, and the organization expanded to work in countries throughout Oceania, Latin America, and Asia (Dutra et al. 2015; Nguyen et al. 2015). MosquitoMate, a US-based start-up, collaborated with Alphabet Inc subsidiary Verily to conduct successful field trials of an Aedes aegypti program in California (Crawford et al. 2020). MosquitoMate's approach uses *Wolbachia* to crash local *A. aegypti* populations and was granted U.S. Environmental Protection Agency (EPA) approval to commercially release *Wolbachia* infected *Aedes albopictus* in 20 states and the District of Columbia after successful field trials in Kentucky, California, and New York (US EPA 2017).

The other genetic approach common to pest management relies on the release of transgenic organisms that limit their own population growth over time. Similar to the bacterial symbiont strategy, the use of self-limiting transgenic organisms has been primarily developed for managing insect populations. Oxitec, a UK-based private company, has been a primary developer in commercializing the use of transgenic *A. aegpyti* male mosquitoes with a repressible dominant lethal gene (Phuc et al. 2007). They have held field trials in the Cayman Islands, Panama, and Brazil, and, in May of 2020, obtained an EPA permit to conduct pilot trials in Florida and Texas after an earlier permit application was withdrawn after the proposed project received significant opposition from local residents in pilot trail communities (Maxmen 2012; Nading 2015; Najjar et al. 2017). In all aforementioned genetic pest management strategies, new organisms have to be introduced into local populations either continu-

ously to suppress the population or sufficiently large a number to establish the strain of *Wolbachia* in the population.

Gene drives, for better or for worse, establish themselves into a population much more readily than other genetic approaches. Gene drives use endonucleases to copy themselves into genomic loci before meiosis so that in the germline, wild-type alleles are converted into drive alleles (Burt 2003; Esvelt et al. 2014). This results in drive alleles being passed on to progeny at rates higher than Mendelian genetics would predict. While first theorized in 2003, the advent of CRISPR technology has rapidly accelerated the development of gene drives in a number of pest species including *Anopheles gambiae, Anopheles stephensi and Aedes aegypti* (Gantz et al. 2015; Simoni et al. 2020; Hammond et al. 2016; Kyrou et al. 2018). Additionally, proof-of-principle experiments in laboratory settings have shown that gene drives could plausibly be made in mouse lines albeit the efficiency homology-directed repair (HDR) of the drive allele would need to be improved to yield sustained population suppression (Grunwald et al. 2019). A Y-linked editor would not rely on HDR after the initial construct is knocked-in to the background species. Moreover, as the Y-linked editor would not drive on its own, its application can be targeted to a specific geographic location whereas a gene drive could not. Notwithstanding, Y-linked editors are able to provide robust population suppression comparable to a female-specific lethal gene that drives in males (Fig. 1-1).

Sex-biasing in males was previously explored in the 1980s, but the genotype was not heritable as it required selectively bred females to produce the sex-biased sons. (McLaren and Burgoyne 1983). This thesis will describe methods for generating a male mouse that would only have male progeny in a manner that is heritable. If successful, this mouse would serve as a proof-of-principle for sex-biasing in mammalian systems and would provide an alternative non-drive approach to using baits, traps, and poisons for suppressing invasive rodent populations.

1.2 Proposed Contribution

The design for a Y-linked editor relies on knocking-out a critical sequence within the female-specific noncoding RNA Xist. The first 900 base pairs of exon 1 of Xist encodes a series of repeated stem-loop motifs, of which four are strictly required for X-inactivation in mice (Wutz, Rasmussen, and Jaenisch 2002). There is no known phenotype for male mice with impaired or lost Xist function nor daughters who inherit a dysfunctional maternal copy of Xist. Daughters who inherit a dysfunctional paternal copy of Xist, however, fail to develop beyond embryonic day 8.5 without X-inactivation in the trophoblast (Marahrens et al. 1997; Hoki et al. 2009). I intend to design a construct an RNA-guided nuclease and multiple guide RNAs targeting sequences flanking or distributed throughout this region to yield targeted deletion and loss of Xist function to arrive at the phenotype of a male sex-biased mouse. I also intend to lay the intellectual foundation for culturally congruent eco-cisgenic bioengineering and provide initial design for a male sex-biased mouse.

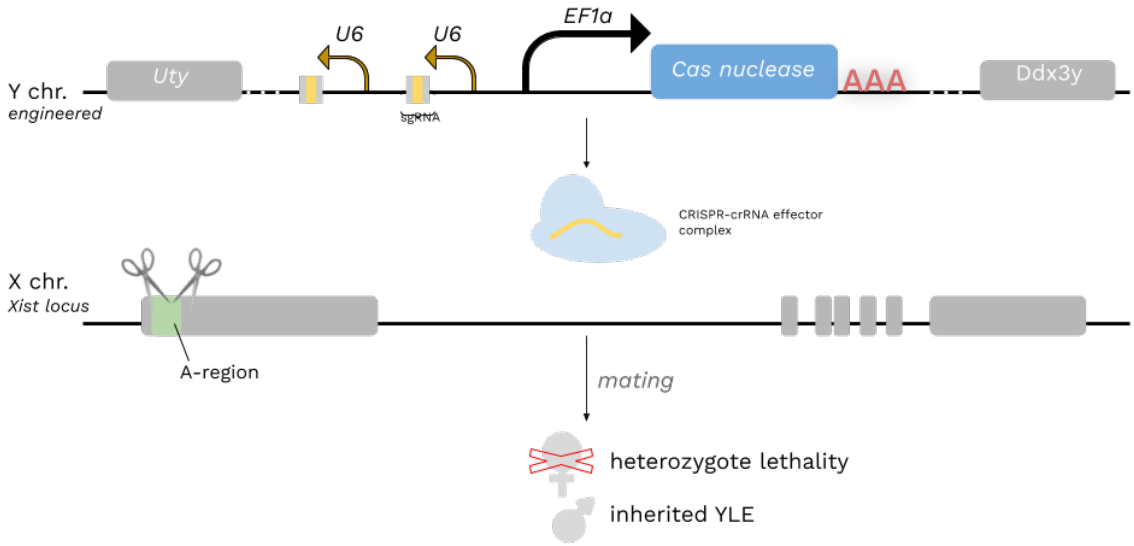

Figure 1-2: Schematic of a Y-linked editor targeting Xist locus. The locus on the Y-chromosome was selected due to prior work indicating robust expression of GFP from a constitutive promoter (Zhao et al. 2019).

Chapter 2

CRISPR Guide Screening

2.1 Overview

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and their associated Cas (CRISPR associated) proteins represent a major leap forward in the development of genomic editing technology (Jinek et al. 2012; Mali et al. 2013). Earlier methods like Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) rely on generating and assembling specific proteins for each dsDNA target site and can be cumbersome and costly to use (Anzalone, Koblan, and Liu 2020). CRISPR/Cas systems were originally found in bacteria where they serve as an RNA-guided adaptive immune response to destroy foreign viral DNA. Type II CRISPR/Cas systems like CRISPR-Cas9, and type V systems like CRISPR-Cas12a (formerly CRISPR-Cpf1), use Watson-Crick base pairing between a single guide RNA (sgRNA) and its target for site-specific DNA recognition and double-strand cleavage which can be repaired either by error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) given an appropriate DNA template (Zetsche et al. 2015, 1; Jinek et al. 2012; Nishimasu et al. 2014). The genetic targetability of a Cas protein is only constrained by the protospacer-adjacent motif (PAM) that corresponds to the Cas protein to be used. In order to ensure efficient disruption of the Xist locus, highly active sgRNAs must be selected. This chapter will describe the methods used to identify and screen potential guide RNA sequences.

2.2 Bioinformatic screen of guides

The selection of CRISPR sgRNAs requires the consideration of a variety of factors including but not limited to: on-target activity, off-target incidence and location, sgRNA hairpin stability, and local chromatin architecture. For this project, we used an automated bioinformatic pipeline to identify and screen sgRNAs for off-targets. *Streptococcus pyogenes* Cas9 (SpCas9) guides were identified using the Breaking-Cas online tool, and then selected for in-vitro activity based on location on the Xist transcript (Oliveros et al. 2016). Six guides were each tested experimentally from both the 5' and 3' end of the A region. All potential Cas12a guides were identified by corresponding PAM location, then scored used CRISPR-DT and CINDEL for initial activity prediction, then analyzed with CasOFFinder for off-target activity (Zhu and Liang 2019; Kim et al. 2017). Cas12a guides with off-targets resulting from fewer than 3 mismatches were dropped unless the off-target was in an intergenic region or in an intron. Guides were found for both wild-type *Lachnospiraceae bacterium* ND2006 (LbCas12a) which uses a 5'-TTTV-3' PAM and the "RR" variant with amino acid substitutions at G532R and K595R in the PAN-proximal region which tolerates an expanded 5'-TYCV-3' PAM (Gao et al. 2017, 1). Of particular interest were guides that disrupted the series of AUCG tetraloop folds which must remain intact for proper dosage compensation (Duszczyk et al. 2011).

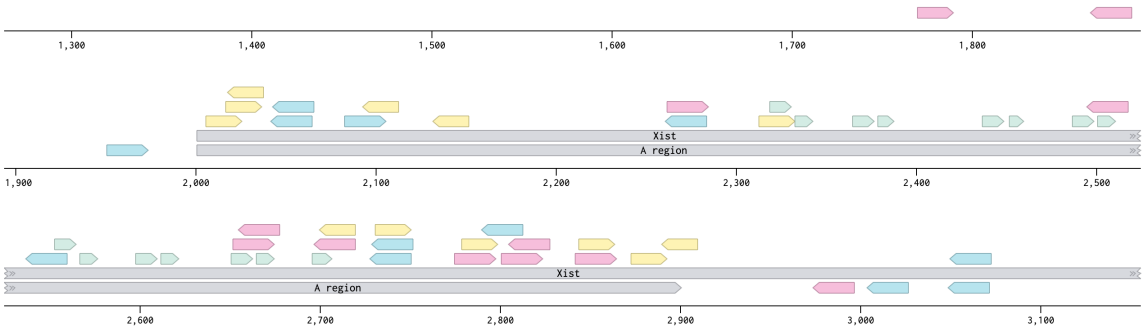

Figure 2-1: Xist Locus with annotated guides. Stem-loop motifs marked in green, WT LbCas12a (TTTV PAM) marked in pink, RR LbCas12a (TYCV PAM) marked in teal, SpCas9 (NGG) guides marked in yellow

2.3 In-vitro analysis of SpCas9 guides

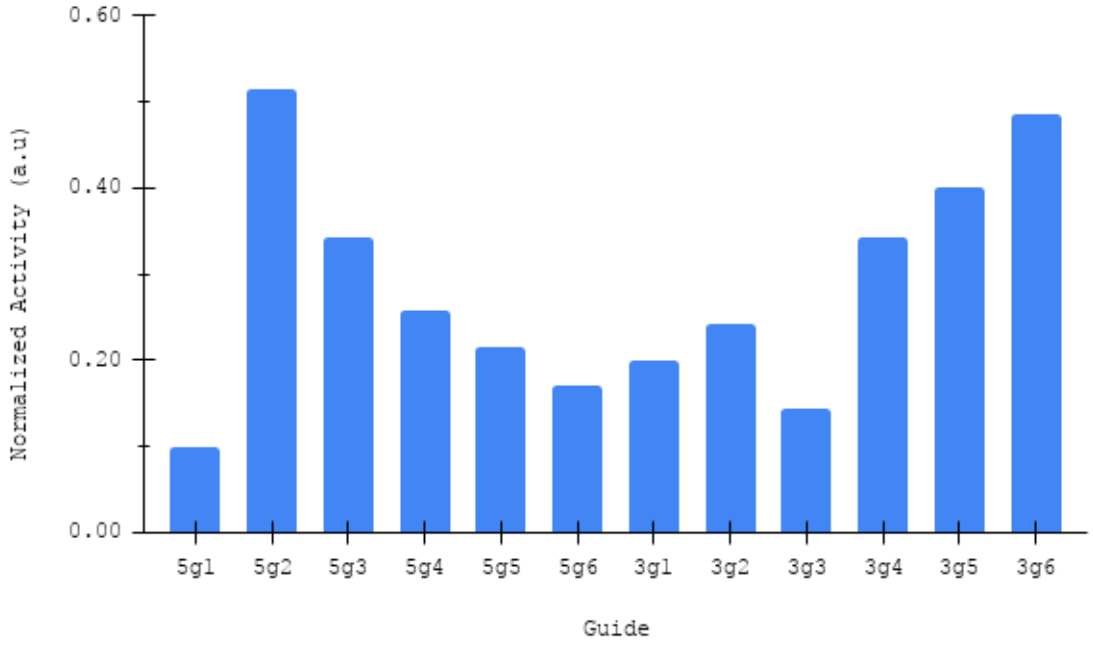

Figure 2-2: Activity of twelve SpCas9 guides in 3T3-L1 cells computed using TIDE. Activity normalized to a control sgRNA for the ROSA26 locus.

To test SpCas9 guide activity in an appropriate cellular context, individual plasmids were constructed with the twelve top sgRNA sequences (See Table 1) from the bioinformatic screen and transfected in duplicate into the murine 3T3-L1 cell line (ATCC® CL-173™) in 6-well plates using Lipofectamine 3000™. Each plasmid contained the M. musculus U6 promoter to drive guide expression and the EF-1a core promoter to drive expression of the SpCas9 nuclease. Cells were also transfected with a CMV-TdTomato plasmid to assess transfection efficiency and allow for FACS sorting. Transfected cells were sorted 48 hours after transfection on a FACS Aria machine (Becton Dickinson) with at least 30,000 gated events measured in the PE-YG channel (561nM laser for excitation, 582/15 filter for detection). TdTomato positive cells underwent genomic DNA extraction using the PureLink™ Genomic DNA mini kit (ThermoFisher). Genomic DNA was amplified using PrimeStar Max (Takara Bio) under standard conditions and Sanger sequenced (Genewiz). Sanger sequences were

analyzed using the Tracking of Indels by Decomposition (TIDE) website (Brinkman et al. 2014). Traces from experimental conditions were compared to an amplicon generated from non-transfected 3T3-L1 cells. Indel formation was normalized to activity on a previously published SpCas9 guide for the "safe-harbor" ROSA26 locus (Romanienko et al. 2016). Two guides had activity at or near fifty percent of the activity of the control guide, and five additional guides had near twenty-five percent activity of the control guide.

With SpCas9 guide on-target activity in hand, the simplest design for a male sex-biased mouse disrupting the Xist locus would consist of Pol II driven expression of SpCas9 and two Pol III promoters driving expression of sgRNA 5g2 and 3g6 (See Fig 2-2).

2.4 Design for Cas12a Multiplexing

A differentiating feature between Cas9 and Cas12a is the ability of Cas12a to process its own sgRNAs from a transcript (Zetsche et al. 2015, 1). From an engineering perspective, this means that multiple genetic sequences can be targeted using a single transcript under a sufficiently processive promoter. This greatly streamlines the design of CRISPR constructs for a wide range of applications, from modeling complex genetic networks or creating combinatorial genetic circuits. Until recently, a bottleneck in the process of generating multi-membered arrays has been the lack of diversity in functional Cas12a crRNA, or scaffold, sequences (Campa et al. 2019; *Strait et al., in preparation*). Highly repetitive crRNAs not only present challenges from a molecular assembly and synthesis standpoint, they also hamper the evolutionary stability of CRISPR systems in vivo. Heritable genome editing tools like gene drives and Y-linked editors need to target multiple loci simultaneously in order to curb the rise of resistant alleles over generational time-scales. Additionally, recombination between highly repetitive regions could abrogate functional array activity.

Here, we present a construct containing LbCas12a (RR) and a 12-membered array under a single, constitutive Pol II promoter (Fig. 2-3). Campa et al. 2019 were able

to induce robust gene activation using 20-member arrays in a VPR assay activating 10 endogenous loci in a mammalian context using the mouse non-coding RNA Metastasis-associated lung adenocarcinoma transcript 1 (MATLA1) which forms a bipartite triple helix (triplex) structure to stabilize nascent mRNA (Fig. 2-3a). Alternatively, the array could have its own Pol II promoter and oriented in the antisense direction to prevent steric interference from recruited polymerases (Fig. 2-3b).

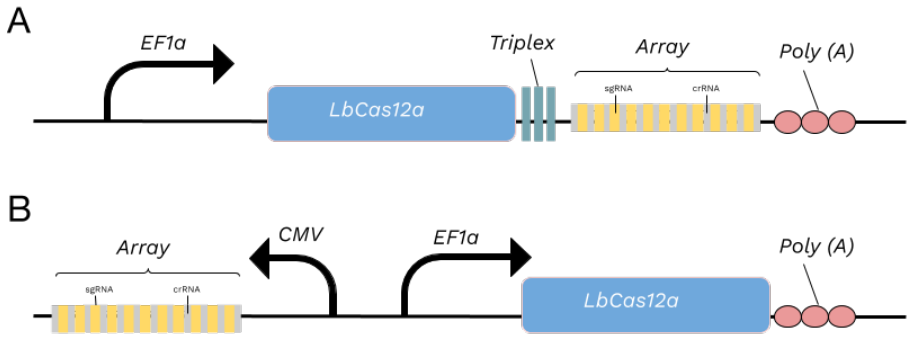

Figure 2-3: Two designs for proof-of-principle constructs using multiplexed arrays. A. Single-transcript expression of the nuclease driven by a EF1a Pol II promoter. B. Two transcript design whereby the guide array and nuclease are each driven by their own polymerase

Chapter 3

Eco-cisgenic Bioengineering

3.1 Background

One of the most prevailing limiting sentiment about the use of transgenic organisms is the concern over the perceived “unnatural-ness” of the genetic modification. Indeed, when comparing the *A. aegypti* programs by MosquitoMate and Oxitec, both functionally arrive at the same technical endpoint, a reduction in the overall population of mosquitos by introducing sterile males. However, they have had strikingly different paths to deployment in Florida. MosquitoMate’s technology, regulated by the EPA, was approved for use rather quietly in 2017. Philip Stoddard, at the time the Mayor of South Miami, the location of the first deployment site in Florida, gave a succinct response to why there was not much controversy: "It’s a lot less controversial because nothing has been genetically modified (Lipscomb 2017; Najjar et al. 2017).” Oxitec, on the other hand, began giving Town Hall meetings to the Florida Keys Mosquito Control District in 2012, yet still rescinded an earlier 2016 permit application after 65% of voters in the town of Key Haven, where the release was to take place, voted against the project in a non-binding referendum (Matthis 2019). Interesting to note, however, that while MosquitoMate’s technology did not introduce transgenes into the genome of the *A. aegypti* population, it did introduce the entire *Wolbachia* genome into the mosquitos. This example is enlightening because while the introduction of foreign nucleic acids on its own was not controversial, the methodology and degree

to which the introduction of foreign nucleic material is perceived as natural played a non-trivial role in public perception.

Another useful example to understand the role of perceived "natural-ness" in genomic modification is the delineation of the terms 'cisgenesis' and 'intragenesis' by the plant breeding field. Both refer to the introduction of genetic elements that come from species that are sexually compatible with the host organism but cisgenesis requires that the introduced gene retains its respective regulatory sequences (promoters, UTRs), orientation, and introns while with intragenesis, the introduced gene can be assembled from different regulatory sequences, with or without introns, in any orientation (Schouten, Krens, and Jacobsen 2006). Cisgenesis also differs from intragenesis because it supposes that the resulting organism could conceivably be generated by conventional plant breeding methods (Schouten and Jacobsen 2008). Significant motivations for the delineation of cisgenesis from intragenesis and transgenesis include faster introduction of beneficial genes into a crop, avoiding linkage drag from conventional breeding whereby a desirable gene might be tightly linked to a deleterious gene which would otherwise be passed along, and perceptual changes to genetic modification both in the eyes of regulatory agencies and the public (Telem et al. 2013).

Survey data from consumers in the United States and Europe suggest that consumers differentiate between transgenic and cisgenic or intragenic food and are more accepting of the latter (Lusk, McFadden, and Wilson 2018). Surveyed consumers are more willing to pay more for cisgenic food over transgenic food and more willing to eat intragenic food yet consumers from different countries can have drastically different willingness to eat intragenic food (Lusk and Rozan 2006; Telem et al. 2013). As these studies primarily come from the field of plant breeding, it remains unclear if the differentiation between cis-, intra-, and transgenic approaches to bioengineering will apply to popular attitudes about genetically modified animals, and, specifically, mammals. A previous series of surveys in Austria and Japan show that respondents do tend to support cisgenesis more than transgenesis, although still strongly believe agricultural products produced cisgenically should be labeled as genetically modified (Kronberger,

Wagner, and Nagata 2013). If the trends from plant breeding continue to carry over to animal breeding, it is likely the relative magnitude of the effect might be smaller in animals because animal-related genetic modifications, broadly, are viewed more skeptically than plant-based applications (Costa-Font, Gil, and Traill 2008).

Nevertheless, our motivation for developing explicitly cis- or intragenic biotechnology is rooted in the belief that communities should have an active role in the development of technology that is used in their community. Other ecological engineering projects in our lab have found that communities do have preferences for how organisms engineered to be released in their environment are constructed (Buchthal et al. 2019). To this end, in the development of a male sex-biased mouse we also investigated how we might engineer systems considering where genetic elements are natively found. While this document presents traditional transgenic techniques and methods for generating our desired phenotypic mouse as a proof-of-principle, I will also present designs for developing an eco-cisgenic mouse with the same desired phenotype. Here, I define eco-cisgenic as containing or using the genetic elements from a host species and/or commensal microbes that serve an essential role in the proper function of the whole organism (Stencel and Proszewska 2018).

3.2 Cisgenic murine Pol III promoters

To ensure sufficient sgRNA production, reliable and robust promoters are necessary to drive sgRNA expression. We were unable to find literature on the activity of the canonical murine U6 promoter used in CRISPR studies compared to other murine U6 sequences so we decided to characterize other murine U6 promoters to potentially find more active variants. To identify additional cisgenic M. musculus Pol III promoters, we conducted a BLAST search of the small nucleolar RNA (snoRNA) that is endogenously expressed by the canonical murine U6 Pol III promoter from chromosome 10. We found exact matches to the same snoRNA sequence at 14 other genomic loci representing 10 different chromosomes. While all shared the same snoRNA sequence, the sequences were highly divergent. We then designed primers to amplify 500 bp

upstream of the repeat region from genomic DNA and subcloned the sequences into a VPR activation circuit.

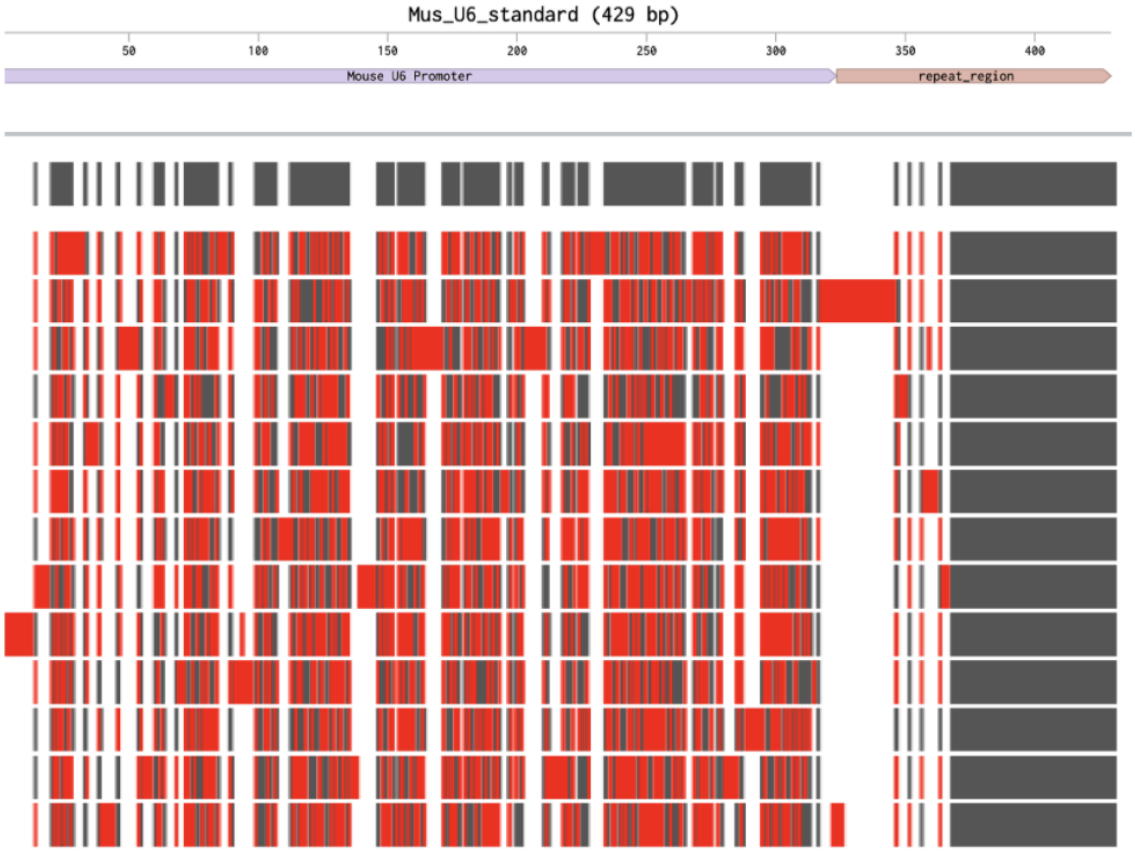

Figure 3-1: Sequence alignment of the 13 putative Pol III promoters. Grey bars indicate regions that match the canonical U6 promoter, red indicates mismatching regions. Alignment generated using MAFFT v7.

The VPR activation circuit uses a fusion protein consisting of a nuclease-dead LbCas12a (dLbCas12) protein linked to the Vp64 transcriptional activator, along with transcription factors p65 and Rta. This fusion protein is able to activate the transcription of a fluorescent protein under a tight TRE promoter via dCas12a binding to the TetO operator (Chavez et al. 2015). In our assay, the sgRNA for TetO is expressed by the putative Pol III promoters, thereby tying fluorescence activity to promoter efficiency. Fluorescence activity is normalized to the activity of the human U6 promoter and a random nucleotide sgRNA driven by the human U6 promoter used as a negative control to capture basal leaky expression in the circuit.

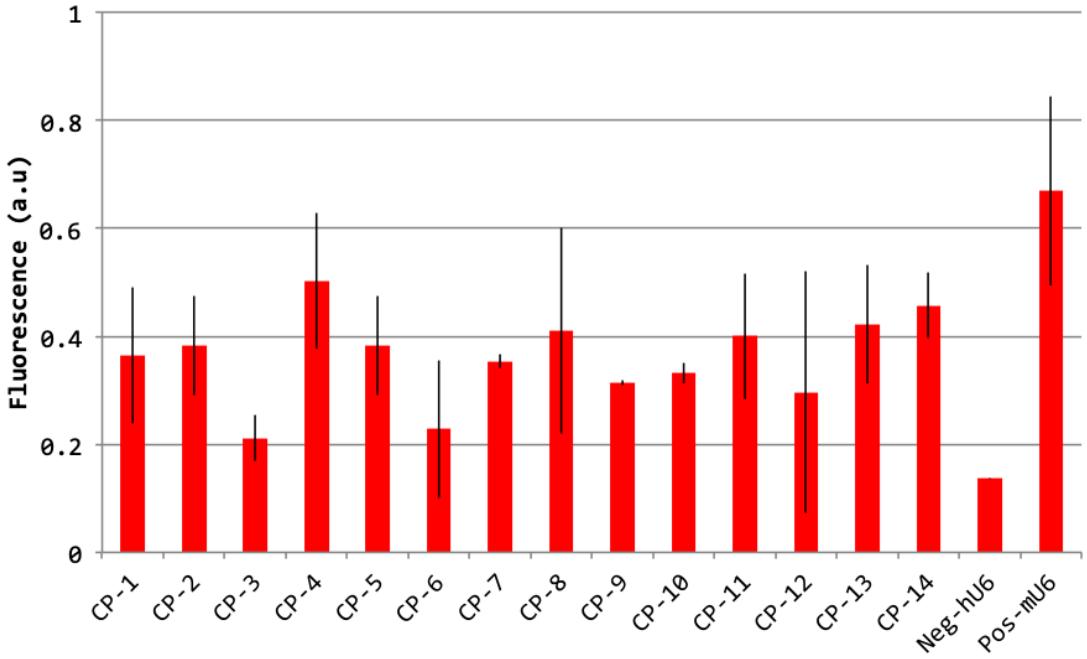

Figure 3-2: Relative fluorescence activity of fourteen cisgenic murine U6 promoters in 3T3-L1 cells in a VPR activation screen. Activity normalized to the fluorescence activity of the human U6 promoter. Data shown is an average of two replicate transfections. Error bars indicate standard error.

From our screen, no cisgenic promoter was as efficient for expressing sgRNA as the canonical U6 promoter from chromosome 10. Several guides were roughly 40 as active. The canonical murine U6 promoter is less efficient than other U6 promoters used in literature, like the human and porcine U6 promoters, along with the *Rattus norvegicus* U6 and 7sk promoters, which would otherwise make it a less than ideal choice for transgenic guide expression (data not shown). While most of the cisgenic promoters are still able to activate the VPR circuit, using them would likely result in a less robust

3.3 Streptococcus Canis Cas9

Streptococcus canis Cas9 (ScCas9), a highly similar Cas9 ortholog, is an attractive choice for an eco-cisgenic design; it shares similar sgRNA architecture as SpCas9, it uses a more expansive 5'-NNG-3' PAM sequence compared to SpCas9's 5'-NGG-3'

PAM, and is able to edit mammalian cells (Chatterjee, Jakimo, and Jacobson 2018). Additionally, *S. canis* is natively found in a variety of mammalian species including mice and rats (Corning, Murphy, and Fox 1991; Iglauer et al. 1991). Sixteen guide sequences (See Table 2) for ScCas9 targeting the Xist locus were identified using the CRISPOR online tool (Haeussler et al. 2016). Guides were selected based on three criteria: their location along the Xist transcript, no off-target matches with an appropriate PAM within 3 or fewer sequence mismatches, and the presence of an optional "T" at the 3' end of its corresponding PAM sequence which has been shown to improve efficiency experimentally (Pranam Chatterjee, personal communication). For this application, a variant of ScCas9, ScCas9(+), with an amino acid substitution in the PAM-interacting domain for improving specificity would be preferred. To experimentally validate these guide sequences, a similar approach to the validation of SpCas9 guides previously mentioned can be used.

3.4 Cas12a orthologs from commensal bacteria

As a Cas12a-based system would be preferred to allow for multiplexing, an ortholog from a murine commensal bacteria would be an ideal choice for eco-cisgenic design. Unfortunately, there is no presently characterized Cas12a ortholog that has met that specification. The *Lachnospiraceae* family is considered ubiquitous in mammals, however, *Lachnospiraceae bacterium* ND2006, now known for its CRISPR system, was found in a study of the rumen of sheep and cattle (Meehan and Beiko 2014; Zetsche et al. 2015, 1). We therefore sought to find other Cas12a orthologs that have been confirmed to be commensal in mice. To do so, I first ran a protein BLAST of the Lb-Cas12a WT sequence on all genomes within the NCBI database. All bacterial species that had Cas12a-like putative proteins were then screened in the publicly available mouse intestinal Bacterial Collection (miBC) hosted by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Lagkouvardos et al. 2016). This search resulted in CRISPR systems retrieved from *Parabacteroides distasonis* and *Bacteroides plebeius*. Next, a literature search was conducted on the

remaining Cas12a-like hits to find any additional eco-cisgenic orthologs. This identified a CRISPR system in *Eubacterium coprostanoligenes* as another mouse commensal bacterium with a CRISPR system (Asano, Yoshimura, and Nakane 2013; Sun et al. 2019). An alignment of the amino acid sequences of all three orthologs is presented in Fig 3-3.

The amino acid sequences of these three novel orthologs varied greatly amongst themselves and other Cas12a orthologs which has been previously reported in other Cas12a surveys (Teng et al. 2019; Zetsche et al. 2019). BpCas12a, EcCas12a, and PdCas12a, also contain the conserved RuvC-like domains, previously identified in the literature, which mediates target DNA cleavage (Zetsche et al. 2015).

An analysis of the crRNA sequences and structures in Fig. 3-4 follow trends previously described of a highly conserved stem structure and AAUU motif which is believed to be critical for efficient crRNA maturation (Zetsche et al. 2019). Presently there is no way to predict if a given Cas12a ortholog will work in a mammalian context other than testing them experimentally. To do so, mammalian-codon optimized versions of these nucleases would be cloned into a suitable expression vector, transfected into a well-characterized cell line like Human Embryonic Kidney (HEK293T) or 3T3-L1 and measured for indel formation at established endogenous loci. For Cas12a ortholog validation, it is common to use guides for endogenous human targets like AAVS1, DNMT1, VEGFA, and CD34, all of which have 5′-TTTV-3′ PAMs which all known Cas12a orthologs can use.

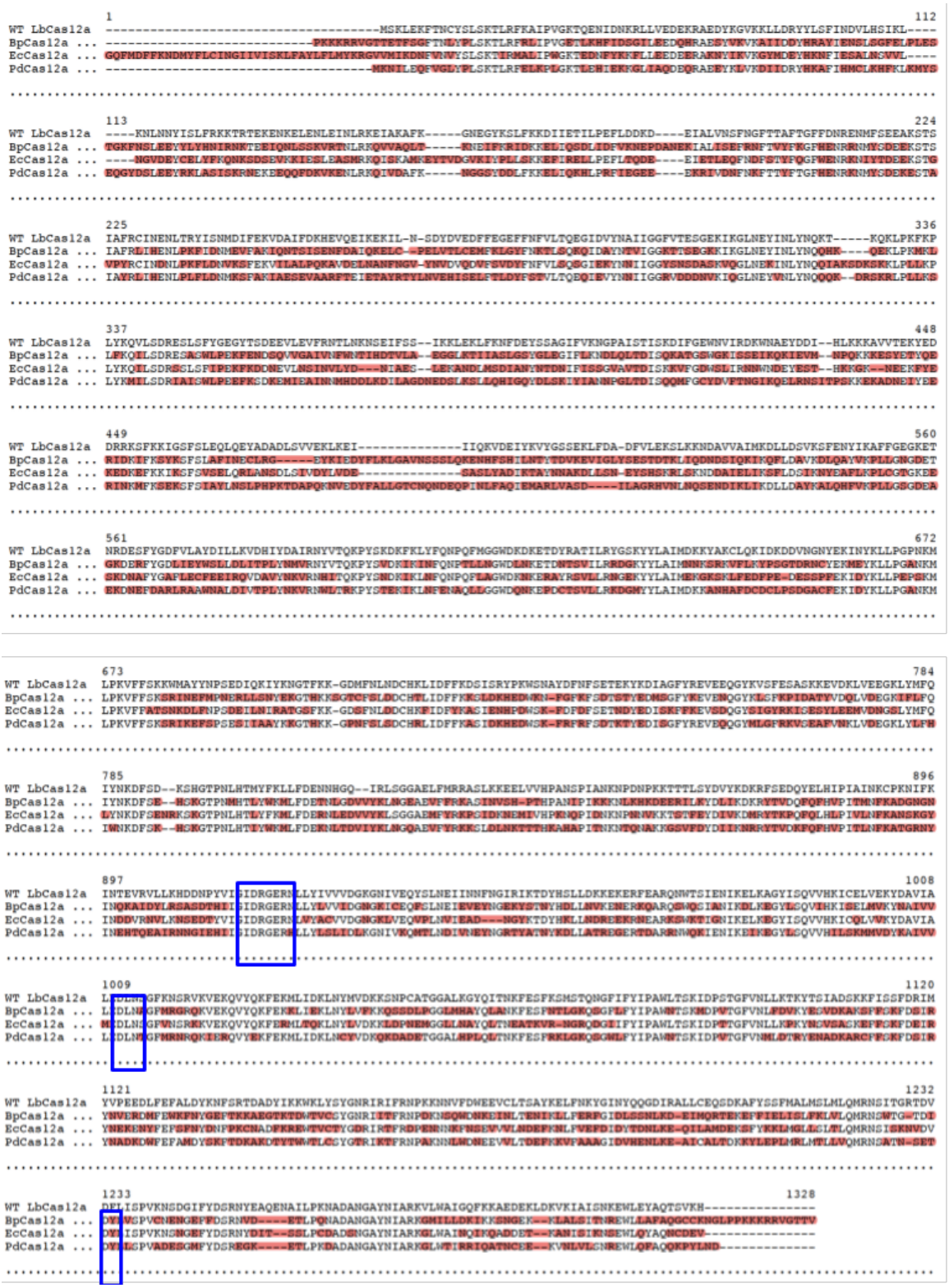

Figure 3-3: Protein Alignment of WT LbCas12, BpCas12a, EcCas12a, and PdCas12a. Conserved sequences are highlighted in red. Catalytic residues from RuvC-like domains are conserved and highlighted in blue.

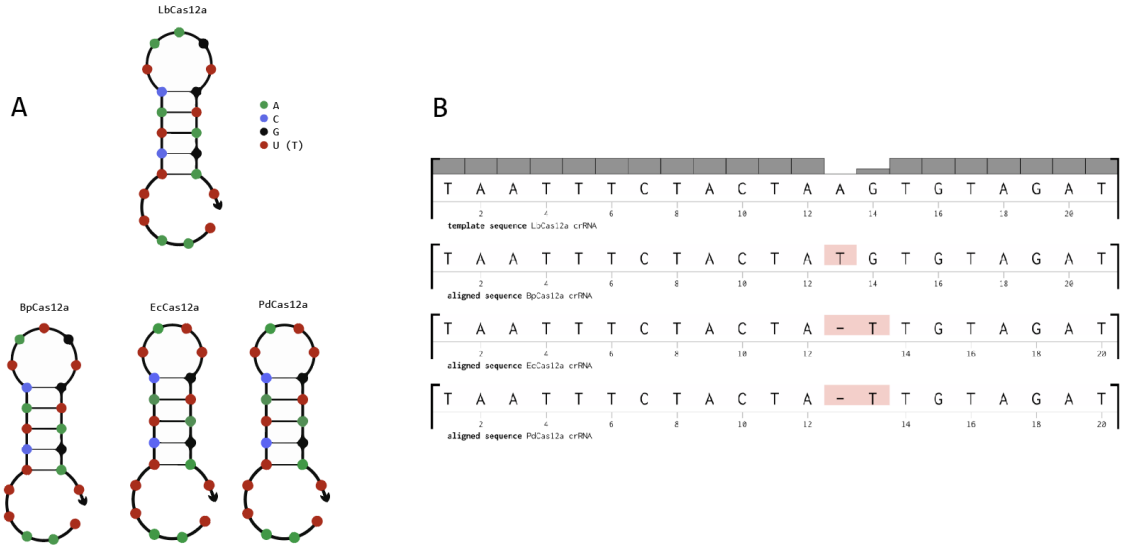

Figure 3-4: Bioinformatic analysis of crRNA from LbCas12a, BpCas12a, EcCas12a, and PdCas12a. A. NUPACK equilibrium base-pairing structures at 37C. B. Nucleic acid alignment of crRNA sequences.

Chapter 4

Perspectives from Two Communities

4.1 Cambridge, Massachusetts

As this technology develops, we would intend to do any potential field trials locally, in Cambridge, Massachusetts USA first. Cambridge is rare in that it has a standing municipal biosafety committee that reviews applications for biotechnology research in the city and has the power to issue permits. The earliest form of this body was the Cambridge Experimentation Review Board (CERB) which was formed in 1976 in response to growing public concern over the expansion of viral and other kinds of genetic research at Harvard University (Feldman and Lowe 2008) . CERB was also notable for including lay residents as well as social scientists and medical doctors to its Board.

Since then, the Cambridge Biosafety Committee, in its various forms, has weighed in on the public safety risks of emerging technology at the forefront of modern biology. To give an example: in 1977 Cambridge was the first US jurisdiction to directly regulate recombinant DNA research (Waddell 1989). As the designs described in this thesis have developed, we have reached out to engage the Committee in what elements they would be interested in the engineering and to understand what kinds of concerns the public-at-large might have. Below is a selection of questions posed at an open-door meeting on November 21, 2019, when presenting the designs for a male sex-biased mouse:

- What happens to the wellbeing of the female rodents as they become more rare?
- How do you determine buy-in to move forward with a field trail?
- Does the State and the Federal government have particular laws and regulations that would play a role in this?
- Have lessons learned from the Florida Oxitec release?
- Is there a phenotypic marker that could be linked to the daughterless system, or for the rodent tracking?
- Is this patentable? Is this going to be seen by the public as something that is going to make someone money?

4.2 Aotearoa (New Zealand)

Rats, stoats, and possums kill approximately 25 million native birds every year in Aotearoa New Zealand. They are the most damaging mammalian predators that threaten the country's natural taonga, or culturally important species, economy, and primary agricultural sector. In response, the government created an ambitious conservation project called Predator Free 2050 (PF2050) which aims to rid the country of invasive mammalian predators by the year 2050. To do so, Aotearoa New Zealand has invested in novel technologies like artificial intelligence-based predator monitoring, long-lived baits, and self-resetting traps, as well as chemical poisons like sodium fluoroacetate or 1080 (Sage and Tabuteau 2020).

The ambitious goal of the project, along with the willingness of the government to invest and use novel pest management solutions stirred debate over potentially using genetic techniques (Morton 2017). As genomic editing technologies for conservation have yet to be tested in the field, such proposals raise questions over the self-determination of indigenous communities as well as the historical legacy of using Oceania as a testing ground for Western scientific interests (Taitingfong 2019). A recent national survey in Aotearoa New Zealand found that segmenting respondents

into groups organized by salient perspective, either humanitarian, individualist, pragmatic, or scientific, underscored the nuances of opinions regarding a panel of genetic pest management techniques (MacDonald et al. 2020).

The reliance on chemical poisons has been a major point of contention between the government and many communities of Māori, the indigenous people of New Zealand (Green and Rohan 2012). For members of the Ngāpuhi nation of Northland region, terms like genetics and species are understood to refer to a relational network that is very different from canonical Western ecology and uses a different visual language. While I can't claim to fully understand the particularities of this Fig 10, it underscores how differing worldviews can lead to differing understandings of how the relationships and organization of the relationships between different members of an ecosystem.

Part of the difference may be understood by the concept of whakapapa, or genealogy, in Māori culture. Whakapapa presents a cultural understanding of how different communities are related and the cultural-historical account of how particular tribes came to the islands of Aotearoa New Zealand. With respect to genetic engineering, a recent paper on Indigenous Perspectives and Gene Editing in Aotearoa New Zealand found that sampled Māori elders defined whakapapa as it relates to gene editing as follows:

> "[whakapapa] was also thought to be connected to the relationality between the species sharing or exchanging DNA. Where DNA associated with genes that are shared in different species is exchanged, this will have less impact on whakapapa. However, if a transferred gene does not have a naturally occurring sequence, then this could be seen to be cutting across whakapapa links. Some felt that gene editing definitely affects whakapapa but that could also be in a positive direction (Hudson et al. 2019)."

While its yet too early to tell what public opinion will be on whether to use genetic control methods like sex-biasing for conservation in Aotearoa, this statement suggests eco-cisgenic approaches might be more favored over tradtional transgenic designs. To end this chapter, I will provide a Tauparapara, or chant, retelling the story of how the

deity Tane obtained the three baskets of knowledge from which mankind was created (Fig 4-2). It provides a counter-narrative to technopostivism, perhaps if the baskets of knowledge to understand the world were placed in Mother Earth, a sex-biased male mouse may raise unique concerns.

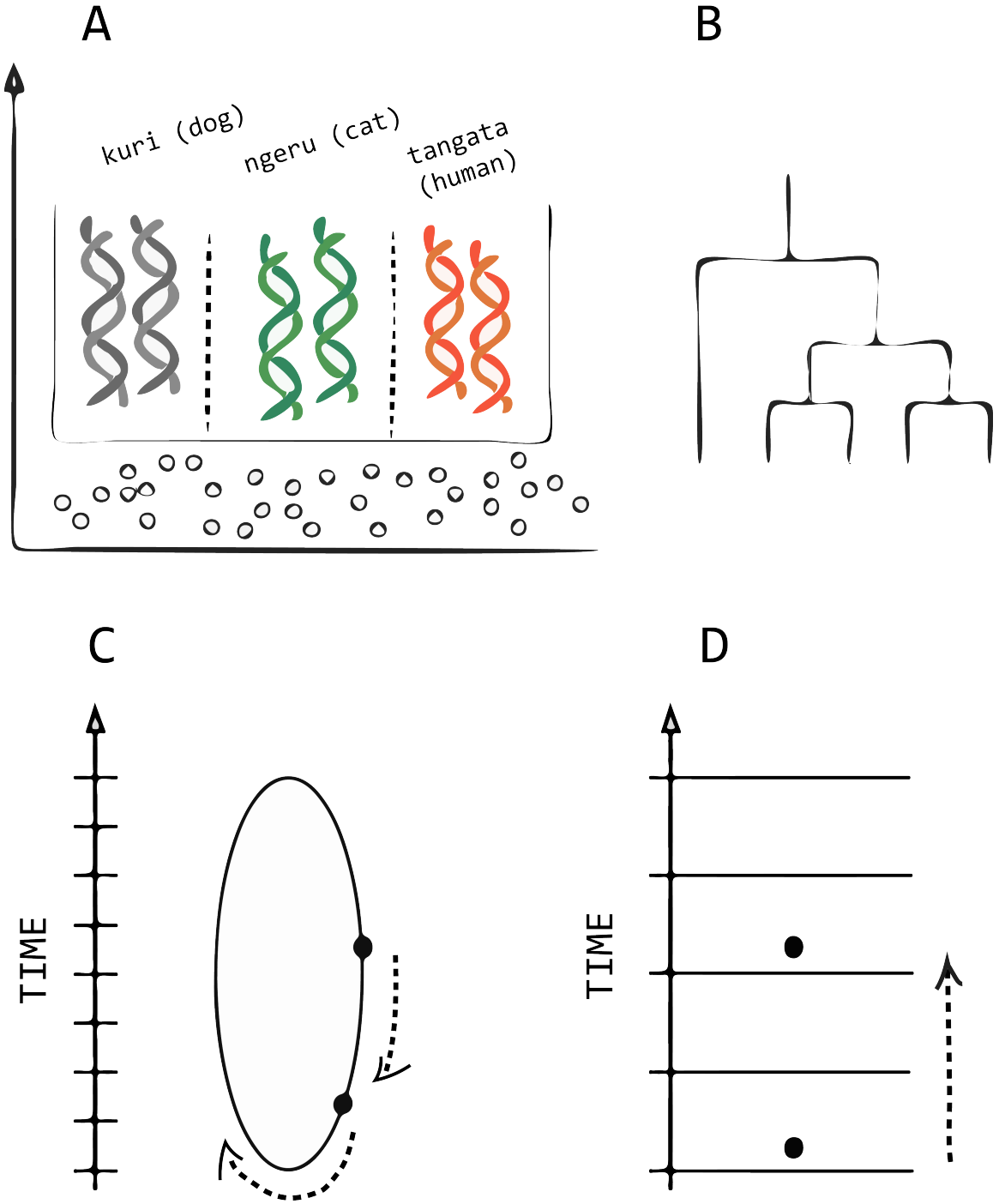

Figure 4-1: Relational differences in how Ngāpuhi and Western tradition view speciation and the progression of time. Ngāpuhi depictions on the left, Western tradition on the right. Reprinted with permission from Bryce Smith, Ngāpuhi elder (Personal Communication)

Tenei au, tenei au	Here am I, here am I
Te hokai nei i taku tapuwae	here am I swiftly moving by
Ko te hokai-nuku	the power of my karakia for swift movement
Ko te hokai-rangi	Swiftly moving over the earth
Ko te hokai o to tipuna	Swiftly moving through the heavens
A Tane-nui-a-rangi	the swift movement of your ancestor
I pikitia ai	Tane-nui-a-rangi
Ki te Rangi-tuhaha	who climbed up
Ki Tihi-i-manono	to the isolated realms
I rokohina atu ra	to the summit of Manono
Ko Io-Matua-Kore anake	and there found
I riro iho ai	Io-the-Parentless alone
Nga Kete o te Wananga	He brought back down
ko te Kete Tuauri	the Baskets of Knowledge
ko te Kete Tuatea	the Basket called Tuauri
ko te Kete Aronui	the Basket called Tuatea
Ka tiritiria, ka poupoua	the Basket called Aronui.
Ki a Papatuanuku	Portioned out, planted
Ka puta te Ira-tangata	in Mother Earth
Ki te whai-ao	the life principle of humankind
Ki te Ao-marama	comes forth into the dawn
	into the world of light
Tihei mauri ora!	I sneeze, there is life!

Figure 4-2: Three Baskets Story in Māori (left) and English (right

Chapter 5

Final Remarks

5.1 Discussion

The introduction of highly invasive species, particularly rodents, into foreign environments was sparked, in no small part, by colonialism and is fueled by globalization and international trade (Hulme 2009). It is imperative than any technology developed in the Global North with evident transferability to the Global South take into consideration the various externalities that might hitch alongside the transfer of technology. Here, the key considerations are of cultural congruence and the need for co-development of technology. Genetically engineering systems can be either transgenic, where DNA is used from different species, or cisgenic, where all DNA used can be found within a given organism (Buchthal et al. 2019; Schouten and Jacobsen 2008). This distinction is important for communities and cultures that practice the ecological species concept, which is to say, organisms are defined by their ecological roles. The second consideration, co-development of technology, is an extension of the consent framework that has historically defined the deployment and development of pest management solutions if communities have been engaged with at all. Beyond simply providing a yes or no on a measure, co-development involves sustained dialogue and collaboration between interested parties.

While rodents and other mammals migrate and travel without respect to political boundaries, a population suppression approach to invasive species management like

this sex-biasing strategy requires the consistent release of engineered organisms to persist. Unlike gene drives which can self-propagate and spread into neighboring populations with limited gene flow, it is not possible for a rearrangement or mutation in a daughterless system to confer an inheritance advantage for the male determining chromosome (Noble et al. 2018). This sex-biasing system is localized by design. This is a deliberate choice to afford greater control and specificity to where and to what degree a population would be suppressed.

Of critical importance to the long-term viability a male sex-biased mouse in the wild is the robust deletion of the Xist region. Inefficient cutting by the CRISPR nuclease could yield unwanted female offspring. For this reason, the ease of multiplexing with Cas12a makes it an endonuclease from this class an attractive choice for future sex-biased mice made using this approach. The Xist locus is well conserved amongst mammals, future sex-biasing strategies based on Xist deletion could extend to other mammalian pests like rats. An alternative, approach to creating a YLE generated sterile daughter would be to use a constitutively active CRISPR nuclease encoded in the same locus as our male sex-biased design to target several female fertility genes for compounding fertility reduction.

5.2 Future Directions

The scope of this thesis is to outline the process for developing a male sex-biased mouse, lay the foundation for an initial proof-of-principle example of a male sex-biased mouse, and introduce the concept of eco-cisgenic bioengineering. While this work has identified key components necessary to make a mouse, much work is still required. The potential guides identified for ScCas9(+) and LbCas12a have yet to be experimentally validated in vitro. Guides for these nucleases have only been bioinformatically identified and would need reliable data suggesting sufficient on-target activity before they can be reasonably expected to yield a reliable phenotype using an approach similar to the one presented here for SpCas9. Moreover, all guides identified in SpCas9, ScCas9(+), and LbCas12a have yet to be tested in a murine cell

line for off-target activity. Unexpected off-target activity could lead to phenotypes that could incur a fitness cost on the resulting mouse line. Genome-wide screens like GUIDE-seq, CIRCLE-seq, would be effective methods to screen for off-target activity. Additionally, the three novel Cas12a orthologs from commensal bacteria have yet to be validated to bind, cleave, and process in a mammalian context. Notwithstanding, the PAM sequences for these novel Cas12a orthologs have yet to be elucidated. It could very well be the case that they have a different set of targetable sequences which may require careful guide consideration to minimize off-target activity. Functional analysis of novel Cas12a orthologs PAM requirements could use a screen like PAM-DOSE or PAM-SCANR (Tang et al. 2019; Leenay et al. 2016).

Chapter 6

References

Alphey, Luke, Mark Benedict, Romeo Bellini, Gary G. Clark, David A. Dame, Mike W. Service, and Stephen L. Dobson. 2010. "Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis." *Vector Borne and Zoonotic Diseases* 10 (3): 295–311. https://doi.org/10.1089/vbz.2009.0014.

Anzalone, Andrew V., Luke W. Koblan, and David R. Liu. 2020. "Genome Editing with CRISPR–Cas Nucleases, Base Editors, Transposases and Prime Editors." *Nature Biotechnology*, June, 1–21. https://doi.org/10.1038/s41587-020-0561-9.

Asano, Krisana, Sayuri Yoshimura, and Akio Nakane. 2013. "Alteration of Intestinal Microbiota in Mice Orally Administered with Salmon Cartilage Proteoglycan, a Prophylactic Agent." *PLOS ONE* 8 (9): e75008. https://doi.org/10.1371/journal.pone.0075008.

Brink, Nico W. van den, John E. Elliott, Richard F. Shore, and Barnett A. Rattner. 2018. "Anticoagulant Rodenticides and Wildlife: Introduction." In *Anticoagulant Rodenticides and Wildlife*, edited by Nico W. van den Brink, John E. Elliott, Richard F. Shore, and Barnett A. Rattner, 5:1–9. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-64377-9_1.

Brinkman, Eva K., Tao Chen, Mario Amendola, and Bas van Steensel. 2014. "Easy Quantitative Assessment of Genome Editing by Sequence Trace Decomposition." *Nucleic Acids Research* 42 (22): e168–e168. https://doi.org/10.1093/nar/gku936.

Buchthal, Joanna, Sam Weiss Evans, Jeantine Lunshof, Sam R. Telford, and Kevin M. Esvelt. 2019. "Mice Against Ticks: An Experimental Community-Guided Effort to Prevent Tick-Borne Disease by Altering the Shared Environment." *Philosophical Transactions of the Royal Society B: Biological Sciences* 374 (1772): 20180105. https://doi.org/10.1098/rstb.2018.0105.

Burt, Austin. 2003. "Site-Specific Selfish Genes as Tools for the Control and Genetic

Engineering of Natural Populations." *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270 (1518): 921–28.
Burt, Austin, and Anne Deredec. 2018. "Self-Limiting Population Genetic Control with Sex-Linked Genome Editors." *Proceedings of the Royal Society B: Biological Sciences* 285 (1883): 20180776. https://doi.org/10.1098/rspb.2018.0776.
Campa, Carlo C., Niels R. Weisbach, António J. Santinha, Danny Incarnato, and Randall J. Platt. 2019. "Multiplexed Genome Engineering by Cas12a and CRISPR Arrays Encoded on Single Transcripts." *Nature Methods* 16 (9): 887–93. https://doi.org/10.1038/s41592-019-0508-6.
Campbell, Karl J., Joe Beek, Charles T. Eason, Alistair S. Glen, John Godwin, Fred Gould, Nick D. Holmes, et al. 2015. "The next Generation of Rodent Eradications: Innovative Technologies and Tools to Improve Species Specificity and Increase Their Feasibility on Islands." *Biological Conservation*, Special Issue on Tropical Island Conservation: Rat Eradication for Species Recovery, 185 (May): 47–58. https://doi.org/10.1016/j.biocon.2014.10.016.
Chapin, F. Stuart, Osvaldo E. Sala, Ingrid C. Burke, J. Phillip Grime, David U. Hooper, William K. Lauenroth, Amanda Lombard, et al. 1998. "Ecosystem Consequences of Changing Biodiversity." *BioScience* 48 (1): 45–52. https://doi.org/10.2307/1313227.
Chatterjee, Pranam, Noah Jakimo, and Joseph M. Jacobson. 2018. "Minimal PAM Specificity of a Highly Similar SpCas9 Ortholog." *Science Advances* 4 (10): eaau0766. https://doi.org/10.1126/sciadv.aau0766.
Chavez, Alejandro, Jonathan Scheiman, Suhani Vora, Benjamin W. Pruitt, Marcelle Tuttle, Eswar Iyer, Shuailiang Lin, et al. 2015. "Highly-Efficient Cas9-Mediated Transcriptional Programming." *Nature Methods* 12 (4): 326–28. https://doi.org/10.1038/nmeth.3312.
Clavero, Miguel, and Emili García-Berthou. 2005. "Invasive Species Are a Leading Cause of Animal Extinctions." *Trends in Ecology & Evolution* 20 (3): 110. https://doi.org/10.1016/j.tree.2005.01.003.
Corning, B F, J C Murphy, and J G Fox. 1991. "Group G Streptococcal Lymphadenitis in Rats." *Journal of Clinical Microbiology* 29 (12): 2720–23. https://doi.org/10.1128/JCM.29.12.2720-2723.1991.
Costa-Font, Montserrat, José M. Gil, and W. Bruce Traill. 2008. "Consumer Acceptance, Valuation of and Attitudes towards Genetically Modified Food: Review and Implications for Food Policy." *Food Policy* 33 (2): 99–111. https://doi.org/10.1016/j.foodpol.2007.07.002.
Crawford, Jacob E., David W. Clarke, Victor Criswell, Mark Desnoyer, Devon Cornel, Brittany Deegan, Kyle Gong, et al. 2020. "Efficient Production of Male Wolbachia -Infected Aedes Aegypti Mosquitoes Enables Large-Scale Suppression of Wild Populations." *Nature Biotechnology* 38 (4): 482–92. https://doi.org/10.1038/s41587-020-0471-x.
Duszczyk, Malgorzata M., Anton Wutz, Vladimir Rybin, and Michael Sattler. 2011. "The Xist RNA A-Repeat Comprises a Novel AUCG Tetraloop Fold and a Platform for Multimerization." *RNA* 17 (11): 1973–82. https://doi.org/10.1261/rna.2747411.
Dutra, Heverton Leandro Carneiro, Lilha Maria Barbosa dos Santos, Eric Pearce

Caragata, Jéssica Barreto Lopes Silva, Daniel Antunes Maciel Villela, Rafael Maciel-de-Freitas, and Luciano Andrade Moreira. 2015. "From Lab to Field: The Influence of Urban Landscapes on the Invasive Potential of Wolbachia in Brazilian Aedes Aegypti Mosquitoes." *PLoS Negl Trop Dis* 9 (4): e0003689.

Esvelt, Kevin M, Andrea L Smidler, Flaminia Catteruccia, and George M Church. 2014. "Concerning RNA-Guided Gene Drives for the Alteration of Wild Populations." *ELife* 3. https://doi.org/10.7554/eLife.03401.

Feldman, Maryann, and Nichola Lowe. 2008. "Consensus from Controversy: Cambridge's Biosafety Ordinance and the Anchoring of the Biotech Industry." *European Planning Studies* 16 (3): 395–410. https://doi.org/10.1080/09654310801920532.

Gantz, Valentino M., Nijole Jasinskiene, Olga Tatarenkova, Aniko Fazekas, Vanessa M. Macias, Ethan Bier, and Anthony A. James. 2015. "Highly Efficient Cas9-Mediated Gene Drive for Population Modification of the Malaria Vector Mosquito Anopheles Stephensi." *Proceedings of the National Academy of Sciences* 112 (49): E6736–43.

Gao, Linyi, David BT Cox, Winston X. Yan, John C. Manteiga, Martin W. Schneider, Takashi Yamano, Hiroshi Nishimasu, Osamu Nureki, Nicola Crosetto, and Feng Zhang. 2017. "Engineered Cpf1 Variants with Altered PAM Specificities." *Nature Biotechnology* 35 (8): 789–92.

Green, W., and M. Rohan. 2012. "Opposition to Aerial 1080 Poisoning for Control of Invasive Mammals in New Zealand: Risk Perceptions and Agency Responses." *Journal of the Royal Society of New Zealand* 42 (3): 185–213. https://doi.org/10.1080/03036758.2011.556130.

Grunwald, Hannah A., Valentino M. Gantz, Gunnar Poplawski, Xiang-Ru S. Xu, Ethan Bier, and Kimberly L. Cooper. 2019. "Super-Mendelian Inheritance Mediated by CRISPR–Cas9 in the Female Mouse Germline." *Nature* 566 (7742): 105–9. https://doi.org/10.1038/s41586-019-0875-2.

Haeussler, Maximilian, Kai Schönig, Hélène Eckert, Alexis Eschstruth, Joffrey Mianné, Jean-Baptiste Renaud, Sylvie Schneider-Maunoury, et al. 2016. "Evaluation of Off-Target and on-Target Scoring Algorithms and Integration into the Guide RNA Selection Tool CRISPOR." *Genome Biology* 17 (1): 148. https://doi.org/10.1186/s13059-016-1012-2.

Hammond, Andrew, Roberto Galizi, Kyros Kyrou, Alekos Simoni, Carla Siniscalchi, Dimitris Katsanos, Matthew Gribble, Dean Baker, Eric Marois, and Steven Russell. 2016. "A CRISPR-Cas9 Gene Drive System Targeting Female Reproduction in the Malaria Mosquito Vector Anopheles Gambiae." *Nature Biotechnology* 34 (1): 78–83.

Hilgenboecker, Kirsten, Peter Hammerstein, Peter Schlattmann, Arndt Telschow, and John H. Werren. 2008. "How Many Species Are Infected with Wolbachia?–A Statistical Analysis of Current Data." *FEMS Microbiology Letters* 281 (2): 215–20.

Hoki, Yuko, Naomi Kimura, Minako Kanbayashi, Yuko Amakawa, Tatsuya Ohhata, Hiroyuki Sasaki, and Takashi Sado. 2009. "A Proximal Conserved Repeat in the Xist Gene Is Essential as a Genomic Element for X-Inactivation in Mouse."

Development 136 (1): 139–46. https://doi.org/10.1242/dev.026427.
Hudson, Maui, Aroha Te Pareake Mead, David Chagné, Nick Roskruge, Sandy Morrison, Phillip L. Wilcox, and Andrew C. Allan. 2019. "Indigenous Perspectives and Gene Editing in Aotearoa New Zealand." *Frontiers in Bioengineering and Biotechnology* 7. https://doi.org/10.3389/fbioe.2019.00070.
Hulme, Philip E. 2009. "Trade, Transport and Trouble: Managing Invasive Species Pathways in an Era of Globalization." *Journal of Applied Ecology* 46 (1): 10–18. https://doi.org/10.1111/j.1365-2664.2008.01600.x.
Iglauer, F., I. Kunstýr, R. Mörstedt, H. Farouq, M. Wullenweber, and S. Damsch. 1991. "Streptococcus Canis Arthritis in a Cat Breeding Colony." *Journal of Experimental Animal Science* 34 (2): 59–65.
Jinek, Martin, Krzysztof Chylinski, Ines Fonfara, Michael Hauer, Jennifer A. Doudna, and Emmanuelle Charpentier. 2012. "A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity." *Science* 337 (6096): 816–21.
Kim, Hui K., Myungjae Song, Jinu Lee, A. Vipin Menon, Soobin Jung, Young-Mook Kang, Jae W. Choi, et al. 2017. "In Vivo High-Throughput Profiling of CRISPR–Cpf1 Activity." *Nature Methods* 14 (2): 153–59. https://doi.org/10.1038/nmeth.4104.
Kronberger, Nicole, Wolfgang Wagner, and Motohiko Nagata. 2013. "How Natural Is 'More Natural'? The Role of Method, Type of Transfer, and Familiarity for Public Perceptions of Cisgenic and Transgenic Modification:" *Science Communication*, August. https://doi.org/10.1177/1075547013500773.
Kyrou, Kyros, Andrew M. Hammond, Roberto Galizi, Nace Kranjc, Austin Burt, Andrea K. Beaghton, Tony Nolan, and Andrea Crisanti. 2018. "A CRISPR–Cas9 Gene Drive Targeting Doublesex Causes Complete Population Suppression in Caged Anopheles Gambiae Mosquitoes." *Nature Biotechnology* 36 (11): 1062–66.
Lagkouvardos, Ilias, Rüdiger Pukall, Birte Abt, Bärbel U. Foesel, Jan P. Meier-Kolthoff, Neeraj Kumar, Anne Bresciani, et al. 2016. "The Mouse Intestinal Bacterial Collection (MiBC) Provides Host-Specific Insight into Cultured Diversity and Functional Potential of the Gut Microbiota." *Nature Microbiology* 1 (10): 1–15. https://doi.org/10.1038/nmicrobiol.2016.131.
Leenay, Ryan T., Kenneth R. Maksimchuk, Rebecca A. Slotkowski, Roma N. Agrawal, Ahmed A. Gomaa, Alexandra E. Briner, Rodolphe Barrangou, and Chase L. Beisel. 2016. "Identifying and Visualizing Functional PAM Diversity across CRISPR-Cas Systems." *Molecular Cell* 62 (1): 137–47. https://doi.org/10.1016/j.molcel.2016.02.031.
Lipscomb, Jessica. 2017. "South Miami to Become Test Ground for Anti-Zika, Wolbachia-Carrying Mosquitoes." May 18, 2017. https://www.miaminewtimes.com/news/south-miami-to-become-test-ground-for-anti-zika-wolbachia-carrying-mosquitoes-9353949.
Lusk, Jayson L., Brandon R. McFadden, and Norbert Wilson. 2018. "Do Consumers Care How a Genetically Engineered Food Was Created or Who Created It?" *Food Policy*, Special issue on The Economics and Politics GM Food Labeling, 78 (July): 81–90. https://doi.org/10.1016/j.foodpol.2018.02.007.
Lusk, Jayson L., and Anne Rozan. 2006. "Consumer Acceptance of Ingenic Foods."

Biotechnology Journal 1 (12): 1433–34. https://doi.org/10.1002/biot.200600187.

MacDonald, Edith A., Jovana Balanovic, Eric D. Edwards, Wokje Abrahamse, Bob Frame, Alison Greenaway, Robyn Kannemeyer, Nick Kirk, Fabien Medvecky, and Taciano L. Milfont. 2020. "Public Opinion towards Gene Drive as a Pest Control Approach for Biodiversity Conservation and the Association of Underlying Worldviews." *Environmental Communication*, 1–15.

Mali, Prashant, Luhan Yang, Kevin M. Esvelt, John Aach, Marc Guell, James E. DiCarlo, Julie E. Norville, and George M. Church. 2013. "RNA-Guided Human Genome Engineering via Cas9." *Science* 339 (6121): 823–26.

Marahrens, Y., B. Panning, J. Dausman, W. Strauss, and R. Jaenisch. 1997. "Xist-Deficient Mice Are Defective in Dosage Compensation but Not Spermatogenesis." *Genes & Development* 11 (2): 156–66. https://doi.org/10.1101/gad.11.2.156.

Matthis, Sara. 2019. "Oxitec Reveals New Technology up for EPA Consideration." Florida Keys Weekly Newspapers. September 11, 2019. https://keysweekly.com/42/oxitec-reveals-new-technology-up-for-epa-consideration/.

Maxmen, Amy. 2012. "Florida Abuzz over Mosquito Plan: Biotech Firm's Bid to Control Dengue Fever Using Genetically Modified Insects Faces Growing Public Opposition." *Nature* 487 (7407): 286–87.

McLaren, Anne, and Paul S. Burgoyne. 1983. "Daughterless X Sxr/Y Sxr Mice." *Genetics Research* 42 (3): 345–49. https://doi.org/10.1017/S0016672300021820.

Meehan, Conor J., and Robert G. Beiko. 2014. "A Phylogenomic View of Ecological Specialization in the Lachnospiraceae, a Family of Digestive Tract-Associated Bacteria." *Genome Biology and Evolution* 6 (3): 703–13. https://doi.org/10.1093/gbe/evu050.

Morton, Jamie. 2017. "Science: What Is Gene Drive Technology and What Does It Mean for New Zealand?" *NZ Herald*, December 4, 2017, sec. New Zealand. https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=11952878.

Nading, Alex M. 2015. "The Lively Ethics of Global Health GMOs: The Case of the Oxitec Mosquito." *Biosocieties* 10 (1): 24–47.

Najjar, Devora A., Avery M. Normandin, Elizabeth A. Strait, and Kevin M. Esvelt. 2017. "Driving towards Ecotechnologies." *Pathogens and Global Health* 111 (8): 448–58. https://doi.org/10.1080/20477724.2018.1452844.

Nguyen, Tran Hien, Hoang Le Nguyen, Thu Yen Nguyen, Sinh Nam Vu, Nhu Duong Tran, T. N. Le, Quang Mai Vien, T. C. Bui, Huu Tho Le, and Simon Kutcher. 2015. "Field Evaluation of the Establishment Potential of WMelPop Wolbachia in Australia and Vietnam for Dengue Control." *Parasites & Vectors* 8 (1): 563.

Nishimasu, Hiroshi, F. Ann Ran, Patrick D. Hsu, Silvana Konermann, Soraya I. Shehata, Naoshi Dohmae, Ryuichiro Ishitani, Feng Zhang, and Osamu Nureki. 2014. "Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA." *Cell* 156 (5): 935–49. https://doi.org/10.1016/j.cell.2014.02.001.

Noble, Charleston, Ben Adlam, George M Church, Kevin M Esvelt, and Martin A Nowak.

2018. "Current CRISPR Gene Drive Systems Are Likely to Be Highly Invasive in Wild Populations." Edited by Michael Doebeli. *ELife* 7 (June): e33423. https://doi.org/10.7554/eLife.33423.

Oliveros, Juan C., Mònica Franch, Daniel Tabas-Madrid, David San-León, Lluis Montoliu, Pilar Cubas, and Florencio Pazos. 2016. "Breaking-Cas—Interactive Design of Guide RNAs for CRISPR-Cas Experiments for ENSEMBL Genomes." *Nucleic Acids Research* 44 (W1): W267–71. https://doi.org/10.1093/nar/gkw407.

Parsons, Barry J., Lesley M. Day, Joan Ozanne-Smith, and Malcolm Dobbin. 1996. "Rodenticide Poisoning among Children." *Australian and New Zealand Journal of Public Health* 20 (5): 488–92. https://doi.org/10.1111/j.1467-842X.1996.tb01627.x.

Phuc, Hoang Kim, Morten H. Andreasen, Rosemary S. Burton, Céline Vass, Matthew J. Epton, Gavin Pape, Guoliang Fu, Kirsty C. Condon, Sarah Scaife, and Christl A. Donnelly. 2007. "Late-Acting Dominant Lethal Genetic Systems and Mosquito Control." *BMC Biology* 5 (1): 11.

Pimentel, David, Lori Lach, Rodolfo Zuniga, and Doug Morrison. 2000. "Environmental and Economic Costs of Nonindigenous Species in the United States." *BioScience* 50 (1): 53–65. https://doi.org/10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2.

Reaser, Jamie K., Laura A. Meyerson, Quentin Cronk, Maj De Poorter, L. G. Eldrege, Edmund Green, Moses Kairo, et al. 2007. "Ecological and Socioeconomic Impacts of Invasive Alien Species in Island Ecosystems." *Environmental Conservation* 34 (2): 98–111. https://doi.org/10.1017/S0376892907003815.

Ritchie, Scott A., Michael Townsend, Chris J. Paton, Ashley G. Callahan, and Ary A. Hoffmann. 2015. "Application of w MelPop Wolbachia Strain to Crash Local Populations of Aedes Aegypti." *PLoS Neglected Tropical Diseases* 9 (7): e0003930.

Romanienko, Peter J., Joseph Giacalone, Joanne Ingenito, Yijie Wang, Mayumi Isaka, Thomas Johnson, Yun You, and Willie H. Mark. 2016. "A Vector with a Single Promoter for In Vitro Transcription and Mammalian Cell Expression of CRISPR GRNAs." Edited by Hodaka Fujii. *PLOS ONE* 11 (2): e0148362. https://doi.org/10.1371/journal.pone.0148362.

Sage, Eugenie, and Fletcher Tabuteau. 2020. "New Transformational Tools for the Predator Free 2050 Effort." The Beehive. July 19, 2020. http://www.beehive.govt.nz/release/new-transformational-tools-predator-free-2050-effort.

Schouten, Henk J., and Evert Jacobsen. 2008. "Cisgenesis and Intragenesis, Sisters in Innovative Plant Breeding." *Trends in Plant Science* 13 (6): 260–61. https://doi.org/10.1016/j.tplants.2008.04.005.

Schouten, Henk J, Frans A Krens, and Evert Jacobsen. 2006. "Cisgenic Plants Are Similar to Traditionally Bred Plants." *EMBO Reports* 7 (8): 750–53. https://doi.org/10.1038/sj.embor.7400769.

Simoni, Alekos, Andrew M. Hammond, Andrea K. Beaghton, Roberto Galizi, Chrysanthi Taxiarchi, Kyros Kyrou, Dario Meacci, et al. 2020. "A Male-Biased Sex-Distorter Gene Drive for the Human Malaria Vector Anopheles Gambiae." *Nature*

Biotechnology, May. https://doi.org/10.1038/s41587-020-0508-1.
Stencel, Adrian, and Agnieszka M. Proszewska. 2018. "How Research on Microbiomes Is Changing Biology: A Discussion on the Concept of the Organism." *Foundations of Science* 23 (4): 603–20. https://doi.org/10.1007/s10699-017-9543-x.
Sun, Jin, Ce Qi, Hualing Zhu, Qin Zhou, Hang Xiao, Guowei Le, Daozhen Chen, and Renqiang Yu. 2019. "IgA-Targeted Lactobacillus Jensenii Modulated Gut Barrier and Microbiota in High-Fat Diet-Fed Mice." *Frontiers in Microbiology* 10. https://doi.org/10.3389/fmicb.2019.01179.
Taitingfong, Riley I. 2019. "Islands as Laboratories: Indigenous Knowledge and Gene Drives in the Pacific." *Human Biology* 91 (3): 179–88. https://doi.org/10.13110/humanbiology.91.3.01.
Tang, Lianchao, Fayu Yang, Xiaoxue He, Haihua Xie, Xiaoyu Liu, Junhao Fu, Haitao Xi, et al. 2019. "Efficient Cleavage Resolves PAM Preferences of CRISPR-Cas in Human Cells." *Cell Regeneration* 8 (2): 44–50. https://doi.org/10.1016/j.cr.2019.08.002.
Telem, R.S., Shabir. H. Wani, N.B. Singh, R. Nandini, R. Sadhukhan, S. Bhattacharya, and N. Mandal. 2013. "Cisgenics - A Sustainable Approach for Crop Improvement." *Current Genomics* 14 (7): 468–76. https://doi.org/10.2174/1389202911314666001 3.
Teng, Fei, Jing Li, Tongtong Cui, Kai Xu, Lu Guo, Qingqin Gao, Guihai Feng, et al. 2019. "Enhanced Mammalian Genome Editing by New Cas12a Orthologs with Optimized CrRNA Scaffolds." *Genome Biology* 20 (1): 15. https://doi.org/10.1186/s13059-019-1620-8.
US EPA, OCSPP. 2017. "EPA Registers the Wolbachia ZAP Strain in Live Male Asian Tiger Mosquitoes." Overviews and Factsheets. US EPA. November 7, 2017. https://www.epa.gov/pesticides/epa-registers-wolbachia-zap-strain-live-male-asian-tiger-mosquitoes.
Veitch, Charles Richard, and Michael Norman Clout. 2002. *Turning the Tide: The Eradication of Invasive Species: Proceedings of the International Conference on Eradication of Island Invasives*. IUCN.
Waddell, Craig. 1989. "Reasonableness versus Rationality in the Construction and Justification of Science Policy Decisions: The Case of the Cambridge Experimentation Review Board." *Science, Technology, & Human Values* 14 (1): 7–25.
Wutz, Anton, Theodore P. Rasmussen, and Rudolf Jaenisch. 2002. "Chromosomal Silencing and Localization Are Mediated by Different Domains of Xist RNA." *Nature Genetics* 30 (2): 167–74. https://doi.org/10.1038/ng820.
Yao, Chenjiang, Zhaoxia Wang, Ying Zhou, Wangjie Xu, Qiaoli Li, Duan Ma, Lianyun Wang, and Zhongdong Qiao. 2010. "A Study of Y Chromosome Gene MRNA in Human Ejaculated Spermatozoa." *Molecular Reproduction and Development: Incorporating Gamete Research* 77 (2): 158–66.
Yao, Chen-Jiang, Wang-Jie Xu, Xiu-Li Gong, Ying Zhou, Zhi-Qiang Yan, Zi-Jue Zhu, Zhao-Xia Wang, Qiao-Li Li, Xin-Bin Guo, and Lian-Yun Wang. 2010. "The Role of Dby MRNA in Early Development of Male Mouse Zygotes." *Asian Journal of Andrology* 12 (4): 567.

Yeap, Heng Lin, Jason K. Axford, Jean Popovici, Nancy M. Endersby, Iñaki Iturbe-Ormaetxe, Scott A. Ritchie, and Ary A. Hoffmann. 2014. "Assessing Quality of Life-Shortening Wolbachia-Infected Aedes Aegypti Mosquitoes in the Field Based on Capture Rates and Morphometric Assessments." *Parasites & Vectors* 7 (1): 58.

Zetsche, Bernd, Jonathan S. Gootenberg, Omar O. Abudayyeh, Ian M. Slaymaker, Kira S. Makarova, Patrick Essletzbichler, Sara E. Volz, et al. 2015. "Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System." *Cell* 163 (3): 759–71. https://doi.org/10.1016/j.cell.2015.09.038.

Zetsche, Bernd, Jonathan Strecker, Omar O. Abudayyeh, Jonathan S. Gootenberg, David A. Scott, and Feng Zhang. 2019. "A Survey of Genome Editing Activity for 16 Cas12a Orthologs." *The Keio Journal of Medicine*.

Zhao, Xiuling, Wei Wei, Hong Pan, Junyu Nie, Dongrong Chen, Pengfei Zhang, Fumei Chen, et al. 2019. "Identification of the Sex of Pre-Implantation Mouse Embryos Using a Marked Y Chromosome and CRISPR/Cas9." *Scientific Reports* 9 (1): 1–9. https://doi.org/10.1038/s41598-019-50731-x.

Zhu, Houxiang, and Chun Liang. 2019. "CRISPR-DT: Designing GRNAs for the CRISPR-Cpf1 System with Improved Target Efficiency and Specificity." *Bioinformatics* 35 (16): 2783–89.