ABSTRACT

SERR, MEGAN ELIZABETH. Towards a Genetic Approach to Invasive Rodent Eradications: Assessing Reproductive Competitiveness Between Wild-Derived and Laboratory Mice (Under the direction of Dr. John Godwin).

Invasive species are of conservation concern, especially on islands that lack natural predators. Over 80% of the world's islands harbor invasive rodents that cause devastating impacts to native flora and fauna. For over fifty years wildlife practitioners and conservationists have removed invasive rodents from islands using rodenticides to help restore island ecosystems. These removals have resulted in substantial conservation gains, but rodenticide-based eradications have drawbacks and are not always popular with the public. Concerns arise because rodenticides are not species-specific, can persist in the environment, have off-target impacts, and raise animal welfare issues. Human-inhabited islands compound these challenges because of human health concerns, and large-scale rodent eradications have only occurred on uninhabited islands. The challenges and limitations to rodenticide-based eradications have prompted a search for alternatives using new genetic technologies.

One technology being explored is the use of a gene drive or selfish genetic element to bias offspring sex ratios and cause extirpation of an island population due to lack of reproduction. Gene drives are systems in which a genetic construct producing a desired phenotype is preferentially inherited by the majority of offspring. To begin addressing the potential efficacy of such an approach, I have examined mating and reproduction using an autosomal selfish genetic element (meiotic distorter) known as the *t*-haplotype. The *t*-haplotype is found exclusively in house mice (*Mus musculus*) and is typically inherited at a rate of 90% or greater from heterozygous sires (homozygotes are sterile). Meiotic drive techniques are being

explored that would use the *t*-haplotype with a genetic construct added to bias offspring sex ratios to causes population reduction.

These techniques would rely on utilizing *t*-haplotype males from the laboratory or breeding this construct into a wild background and the resultant males then mating successfully with wild females. My dissertation has focused on studying the mating behavior and reproductive success of wild-derived and *t*-haplotype laboratory mice to characterize differences in reproduction and male competition. The first set of experiments aimed to confirm that *t*-haplotype laboratory mice could breed with wild-derived mice. The resulting offspring were then assessed for reproductive characteristics and mating success in laboratory settings. My second objective aimed to determine if *t*-haplotype carrying males could reproduce successfully when in competition with wild-derived males, in more natural settings. I found that wild-lab hybrid males were stronger competitors than both purely laboratory strain males and, interestingly, also purely wild-derived males. These results support the feasibility of a *t*-haplotype approach for population eradication of invasive house mice. My research provides an important step in addressing the applied question of whether it is possible to create an engineered gene drive mouse that will be competitive with wild mouse populations. My results also inform reproductive questions about wild-derived and lab mouse behavior and sexual selection in larger more natural settings.

The final aspect of my dissertation work utilized scenario analysis to organize insight and gauge risks in the potential for rodent eradications on inhabited and uninhabited islands by comparing traditional rodenticides with genetic sex-biasing techniques. While my studies have helped determine that (wild-lab) hybrid mice may be strong competitors, it does not answer the question of whether or not wildlife managers should use this technique for removing invasive rodents from islands. The creation of a sex-biasing mouse for eradication involves more than the

technique itself and requires further ecological, social, and ethical explorations into using gene drives for conservation.

Towards a Genetic Approach to Invasive Rodent Eradications: Assessing Reproductive Competitiveness Between Wild-Derived and Laboratory Mice

by
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DEDICATION

To my wonderful husband, for all your encouragement and support.

BIOGRAPHY

Megan Elizabeth Serr (formerly Tracey) grew up in the Coachella Valley, California, where she developed a love for amphibians and reptiles. Since she was seven years old, she knew she wanted to be a biologist and has maintained that passion all her life. She was the first Junior Docent at the Living Desert Zoo and Gardens and thanks her family and early mentors for helping her seek out novel animal adventures. In 1997, Megan enrolled at College of the Desert, where her father worked, and she completed her A.A. degree in 2002. From there, she transferred to California State University San Bernardino, where she completed her B.S. in Biology in 2004.

After graduation, having had a difficult high school experience, she decided she wanted to become a public high school teacher to help those struggling. Over the course of her teaching career, Megan has taught biology, earth science, and environmental science both to traditional and alternative students. In 2008, Megan married and moved to North Carolina, where she continued to teach high school. While Megan loved teaching, she felt unfulfilled in her research and began her masters exploring the demasculinization of Northern Leopard Frogs (*Lithobates pipiens*) exposed to endocrine disrupting compounds. In 2010, she earned her M.S. in Biology through the University of Nebraska at Kearney. After completing her masters, Megan went on to serve as a Laboratory Teaching Technician at North Carolina State University. In the fall of 2013, Megan enrolled in an interdisciplinary Ph.D. program, working for her first time on mammals. However, Megan's passion for herpetology persisted, and she served as the secretary for the North Carolina Herpetological Society, and performed education and outreach in her spare time during her Ph.D. program. Megan loves travelling, kayaking, and herping. Megan plans to continue in academia with the goal of teaching at an institute that balances research and teaching.

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Introduction

Threatened Island Biodiversity

Islands are unique terrestrial ecosystems with insular habitats that have long served as models for studying ecological and evolutionary processes (Graham et al., 2017). More than fifty years after MacArthur & Wilson's (1963) seminal paper on island biogeography, we have come to recognize that, while islands have a higher number of species and endemicity compared to the mainland, they are also more likely to have endangered and extinct species (Warren et al., 2015; Spatz et al., 2017). Islands only make up 5% of all of Earth's landmass, but over 20% of all terrestrial animal species are found on islands, including 41% of all highly threatened terrestrial vertebrates (Tershy et al., 2015; Spatz et al., 2017). This threat to biodiversity is especially pronounced due to the presence of invasive species (Spatz et al., 2017; Bellard et al., 2016) and more than 90% of avian extinctions have occurred on islands, mainly due to predation by invasive mammals (Atkinson, 1985; Steadman, 1995; Bellard et al., 2016; Doherty et al., 2016). Importantly, however, not all is lost in terms of island conservation as Spatz and colleagues have estimated that, where invasive vertebrates are present, management efforts and restoration could help the survival of 39% of all highly threatened vertebrates (2017).

Invasive Rodents on Islands

More than 80% of the world's islands harbor invasive rodents, and these invaders have been implicated in numerous extinctions worldwide (Towns et al., 2006; Caut et al., 2008; Campbell et al., 2015). Invasive rodents were introduced via human travels, often as stowaways on ships (Howald et al., 2007). The most prevalent invasive rodents on islands are rats (*Rattus spp.*) and house mice (*Mus musculus)* (Towns et al., 2006). Rats and mice are omnivorous and can readily adapt to new habitats and climates (Towns et al., 2006; Howald et al., 2007). Islands

often lack native mammalian predators and thus, rodents can cause devastating impacts when introduced (Mulder et al., 2008; Drake & Hunt, 2009). On Lord Howe Island in Australia, invasive rodents impact populations of over 70 plant and animal species and have been linked to the extinction of two plant, thirteen invertebrate, and two vertebrate species (Wilkinson & Priddel, 2011). As islands are often the nesting and breeding grounds for seabirds, rodents can be particularly damaging in this context (Ruffino et al., 2015). Rodents can consume eggs and chicks and attack adults (Fukami et al., 2006; Caut et al., 2008; Caravaggi et al., 2018). Consistent with these documented impacts, the removal of invasive rodents from islands has directly benefited not just seabirds, but other flora and fauna as well (Capizzi et al., 2014; Jones et al., 2016). There can also be indirect beneficial effects of rodent removal. For example, coral reefs surrounding islands can benefit by added nitrogen from the eradication of invasive rodents (Graham et al., 2018) and on Palmyra Atoll, when invasive rats were eradicated, the invasive Asian tiger mosquito (*Aedes albopictus*) was also extirpated (Lafferty et al., 2018).

House Mice on Islands

House mice can readily adapt to a broad range of non-commensal habitats and environments, making them particularly suited for invasion (Bronson, 1984; Mackay et al., 2007; Singleton & Krebs, 2007). This ability to readily establish and adapt makes them the second most widespread mammal on the planet, second only to humans (Mackay et al., 2007; Singleton & Krebs, 2007; Angel et al., 2009). The adaptability and behavior of wild house mice depends on a multitude of factors including, but not limited to, population genetics (Baker, 1981), seasonality (Breakey, 1963; Singleton & Krebs, 2007), food supply (Meikle & Drickamer, 1986), social structure (Singleton et al., 2001; Perony et al., 2012), reproduction (Coppola & Vandenbergh,

1987; Vandenbergh, 1987), and predation (Dickman et al., 1991; USFWS, 2013). For example, on islands uninhabited by humans, grain is generally not available and the main dietary component for mice is invertebrates, contributing to biodiversity loss (Le Roux et al., 2002; Singleton & Krebs, 2007). A lack of predators such as owls, foxes, and snakes may also influence the population ecology of mice on islands (Singleton & Krebs, 2007). When there are ample resources and a general lack of predators, mice reproduce quickly and their populations can grow dramatically in size (Singleton & Krebs, 2007; USWS, 2013). Further, depending on the island's location, seasonality can also play a role in the population size of mice and numbers may dwindle for a period of time only to rise again when conditions permit (Singleton et al,. 2001; Singleton & Krebs, 2007).

Rodenticide-Based Eradication Approaches

The main method for the removal of invasive rodents from islands larger than 5ha is the use of baits laced with rodenticides that are spread aerially (Campbell et al., 2015). Modern rodenticides are second-generation anticoagulants (Capizzi et al., 2014) and the most commonly used and effective second-generation rodenticide is brodifacoum (Howald et al., 2007; Parkes et al., 2011). The use of helicopters to spread rodenticide has allowed for an increase in the number and size of the islands on which this eradication approach can be applied, the largest to date is South Georgia island (total area 30,000 ha) (Simberloff et al., 2018). Worldwide, there have been more than 650 rodent eradications on islands with 80% being successful in eradicating the target rodent species (Holmes et al., 2015).

While rodenticides are effective for eradication, they do raise several concerns and limit where this approach is suitable. One concern in the use of rodenticides is that they are not

species-specific, and off-target impacts, and death can occur when native species ingest baits (Parkes et al., 2011; Campbell et al., 2015; Rueda et al., 2016). Rodenticides have also been found to persist in the environment and bioaccumulate within the food-chain (Masuda et al., 2015; Regnery et al., 2018). Furthermore, rodents can develop resistance to rodenticides rendering them ineffective (Goulois et al., 2017; Berny et al., 2018). In addition to the concerns listed above, a critical limitation to the use of rodenticides on most islands where invasive rodents represent a biodiversity threat is the presence of humans. Consequently, aerial broadcast methods have only been used on uninhabited islands as there are significantly increased challenges and safety precautions needed when livestock, pets, and humans may potentially come into contact with rodenticides (Oppel et al., 2011; Glen et al., 2013; Campbell et al., 2015). Because of the concerns described above, a major challenge remains as an estimated 85% of islands with invasive rodents remain difficult if not impossible to perform eradications on with current methods (Campbell et al., in press). Lastly, there is the issue of public perception of the use of rodenticides (Fitzgerald, 2009; Cowan & Warburton, 2011). The concerns voiced include questioning the humaneness and effectiveness of rodenticide control methods, their unknown environmental impacts, the inability to target specific animals, and their cost of use (Dubois et al., 2017; Fitzgerald, 2009; Reiter et al., 1999; Holmes et al., 2015). Opponents have also expressed strong ethical concerns about "killing for conservation" and generally prefer no-kill methods (Courchamp et al., 2017). The drawbacks and limitations to rodenticides, along with ethical concerns about their use, have led to the exploration of new eradication technologies (Campbell et al., 2015; Goldson et al., 2015; Sutherland et al., 2018).

Genetic Pest Management

The use of a genetic technique could represent an attractive alternative to rodenticide use on islands. Researchers conceptualized genetic pest-management strategies in the 1950s for insect agricultural pests. The first genetic strategy deployed, and still in use today, is the Sterile Insect Technique (SIT; Knipling, 1955). This technique requires irradiating and releasing large numbers of sterile males into wild populations. When females mate with sterile males, they do not produce offspring, which causes the population to crash (Knipling, 1955; Mumford, 2005). In 1966, the United States implemented SIT for the eradication of the screwworm fly (*Cochliomyia hominivorax*) and this effort has saved US agriculture billions of dollars in livestock losses (Mumford, 2005). Building on the basic SIT approach, the company Oxitec uses genetic engineering to create sterile males with a focus on controlling populations of mosquitoes that are vectors for human diseases (Capurro et al., 2016).

Recently, there has been strong interest in the use of gene drive systems for genetic pest management. First conceptualized by Burt (2003) and expanded upon by others (Sinkins & Gould, 2006; Esvelt et al., 2014), gene drives are a genetic construct that is preferentially inherited by offspring. As the majority of offspring will inherit the genetic construct, modeling suggests such gene drives could spread quickly through a population even if they have a fitness cost (Harvey-Samuel et al., 2017). A gene drive-based approach to pest eradication could use either a naturally occurring drive or a synthetic drive such as CRISPR/Cas9, the latter of which has already been demonstrated in mosquitoes, flies, and yeast (Harris et al., 2012; Dicarlo et al., 2015; Gantz & Bier, 2015). Theoretically, by biasing offspring sex ratios heavily towards one sex, reproduction could be impaired, and the population reduced (Backus & Gross, 2016; Prowse et al., 2017). Scientists are looking at several genetic strategies that could achieve island

eradication using gene drives (Campbell et al., 2015; Leitschuh et al., 2017; Piaggio et al., 2017), one of which is examined in the next section.

t-Sry Technique

The *t*-haplotype is a naturally-occurring and well-studied meiotic drive found in house mice (Silver, 1993).The *t*-haplotype is a series of inversions that collectively represent approximately one third of chromosome 17 (Silver, 1993; Lyon, 2005). The *t*-haplotype impacts sperm motility in males and works as a poison/antidote system. The presence of the *t*-haplotype in heterozygotes decreases the motility of non-*t* bearing sperm and consequently *t*-bearing sperm are transmitted at a much higher rate. Some *t* variants show transmission ratio distortion rates of over 90% (Bauer et al., 2005; Baker, 2008). Because the *t*-haplotype affects sperm and not eggs, transmission in females is unaffected (Lyon, 2003). We are investigating the use of a variant of the *t*-haplotype termed the t^{w2} that transmits at a rate greater than 94% (Silver, 1993). The t^{w2} is originally from a wild background from the East Coast of the USA, but was bred into laboratory mice in 1946 (Dunn & Morgan, 1953). Homozygosity of the *t*-haplotype (t/t) is typically lethal, but this is not true of the t^{w2}, although homozygosity does cause sterility with this variant (Dunn & Levene, 1961; Lyon, 2003). Despite its high rate of inheritance, it is estimated that less than 20% of individuals in wild populations harbor any form of the *t*-haplotype, possibly due to female preference for males lacking the *t*-haplotype (Ardlie & Silver, 1998; Carroll et al., 2004; Lindholm et al., 2013; Manser et al., 2015).

One approach to creating a sex bias in a population for eradication purposes would involve inserting a masculinizing gene into the *t*-haplotype. In mammals, the *Sry* gene (sex-determining region Y) is necessary and sufficient to initiate male development (Gubbay et al.,

1990). A previous study in house mice demonstrated that inserting the *Sry* gene into the autosome of a chromosomal female (XX) mouse resulted in a male phenotype (Koopman et al., 1990). The XX/*Sry* mice, while sterile, were anatomically and behaviorally indistinguishable from the other males. Based on this *Sry* effect, efforts are underway to insert the *Sry* gene into the *t*-haplotype (Kanavy, 2018). This *t-Sry* construct could theoretically create heavily male biased populations as the *t-Sry* males pass the construct down to a high proportion of their offspring. XY/*t-Sry* individuals should be fertile males while XX/*t-Sry* individuals are predicted to be phenotypically male but sterile (Backus & Gross, 2016; Leitschuh et al., 2017; Piaggio et al., 2017).

Reproduction and Male Competition in Laboratory and Wild-Derived House Mice

For a genetic pest management approach to be effective, it is critical that the introduced animals are able to effectively compete for mates, as this will allow the genetic construct to introgress into and then spread in the targeted island population. As the *t-Sry* approach would rely on male carriers, male mate competition is of particular interest. To begin addressing this requirement, I focused on two mouse strains in my dissertation project, a wild-derived island strain and a laboratory line that is the focus of engineering efforts. The following section summarizes some key characteristics of these lines and information relevant to mate competition in mice and their behavior under more naturalistic conditions.

Farallon Island Mice

The Farallon islands are a small group of islands located about 30 miles off the California coast (37°41’ N, 123°0’ W) (Schoenherr et al., 2003). They are a part of the Farallon National

Wildlife Refuge which is protected and has strictly limited access for wildlife researchers only (USFWS, 2013). Coined the 'Galapagos of the North', the islands host the largest breeding seabird colony in the continental United States and are a sanctuary for marine mammals (USFWS, 2013). The islands' late summer maximum temperatures are 14°C, with winter lows reaching down to around 0°C (Schoenherr et al., 2003). During the summer months, dense fog is present while rainfall (~63 cm annually) occurs primarily during the winter months (Schoenherr et al., 2003). Invasive house mice are the only terrestrial mammals currently inhabiting the island and are believed to have been introduced by humans with the construction of a lighthouse in 1855 (Schoenherr et al., 2003; USFWS, 2013).

The Farallon island mice show cyclical variation in population sizes with peak densities occurring in late summer and early fall (USFWS, 2013). The highest densities ever recorded in non-commensal natural habitats have been on Southeast Farallon Island, reaching approximately 1300/hectare (Seamons, 2013; USFWS, 2013). A study reported that their diet consists primarily of invertebrates (Jones et al., 2006). These mice present direct and indirect threats to conservation and the United States Fish and Wildlife Service is planning for eradication of this population with rodenticide (USFWS, 2013). Genetic analyses of the mice show their subspecies is the Western European (*M.m. domesticus*) and they do not carry the *t*-haplotype (Morgan et al., 2018).

Laboratory Mice

House mice are the most commonly utilized mammal for laboratory research (Battey et al., 1999). Laboratory mouse strains have been artificially housed and have undergone both deliberate and inadvertent selection under these captive conditions (Silver, 1995; Morse, 2007).

As such, laboratory mice often do not behave as their wild counterparts do because they were derived from domesticated stocks and then typically inbred for many generations to create genetically homogenous stocks (Silver, 1995; Morse, 2007). Standard housing is limited to a few rodents per cage, which are typically no larger than 29cm wide x 40cm long x 19cm high (Fawcett & Rose, 2012). As early as the 1950's, scientists began to consider the effects of laboratory housing and began to explore mouse behavior and social structures in more naturalistic settings (Beach, 1950; Baker, 1981; Beckers et al., 2009; Didion & De Villena, 2013) and these studies often produced results that contrasted with laboratory studies (Fawcett & Rose, 2012). For example, it was determined that mice raised in laboratory conditions have more restricted patterns of movement (Perony et al., 2012), fewer hierarchical relationships (Howerton et al., 2008; Chalfin et al., 2014), and altered patterns of reproduction (Drickamer, 1982; Singleton et al., 2001). In terms of reproduction, previous research demonstrated that laboratory housing may delay puberty (Drickamer, 1982; Coppola & Vandenbergh, 1987), reduce litter sizes (Bronson, 1984; Singleton & Krebs, 2007), and skew sex ratios (Meikle & Drickamer, 1986).

Mouse Reproduction and Male Competition

Despite laboratory mice being one of the best studied animals, the behavior and interactions of house mice in more naturalistic settings are still not well understood. Furthermore, research investigating interactions between wild-derived mice and laboratory mice is very limited, presumably because this has not been an important question prior to considering genetic pest management for mice. The ability of a gene drive approach biasing offspring sex ratios to achieve rodent eradication on islands requires a more thorough understanding of these

interactions. Likewise, a requirement of the *t-Sry* sex biasing approach is ensuring that *t*-haplotype carrying males are reproductively successful when released on an island. Thus, a primary aim of my dissertation research was to determine the reproductive and competitive differences between wild-derived and laboratory mice.

Both the Farallon mice and the t^{w2} bearing laboratory mice used in our studies are of the same subspecies (*Mus musculus domesticus*), though to our knowledge there have been no attempts to mate the two together (Farallon wild-derived and laboratory mice). Prior to our studies, we were unsure if speciation and sexual selection had caused reproductive isolation between the different mouse populations (strains). Lack of mating and or sterile offspring are common among subspecies of house mice. For example, previous research found that *M.m. domesticus* and *M.m. musculus* have been found to hybridize only in certain regions and subspecies matings often produce sterile progeny in laboratory settings (Forejt & Ivanyi, 1974; Turner et al., 2012). Likewise, another study found that male infertility was responsible for non-viability in many of the strains generated in the mouse collaborative-cross genetic reference panel (Shorter et al., 2017).

Chapter two of my dissertation examines the mating of Farallon and t^{w2} laboratory mice in standard laboratory cages as well as differences in reproductive output. Additionally, I examined the outcome of competition for access to females between male wild-derived Farallon and t^{w2}-bearing lab mice. To use the *t-Sry* construct, it is critical to assess male wild-derived, lab, and wild-lab crosses (hereafter hybrid) in a mate competition context. Several previous studies indicate female choice against *t*-haplotype bearing males (Lenington, 1983; Lenington et al,. 1994; Carroll et al., 2004; Manser et al., 2015) as well as a lack of competitiveness for *t*-bearing sperm (Sutter & Lindholm, 2015). House mice are polyandrous in wild and semi-natural

populations with litters typically showing evidence of multiple paternity (Potts et al., 1991; Dean et al., 2006; Firman & Simmons, 2008). Males also compete for access to females and the establishment of the deme (Latham & Mason, 2004; Bonhomme & Searle, 2012). Male mice are territorial, and fighting is common when they encounter other males (Novotny et al., 1990; Latham & Mason, 2004), with dominant males siring more offspring by preventing subordinate males from accessing females (Novotny et al., 1990). To begin assessing the outcome of male competition between wild, laboratory, and hybrid wild-lab strains in chapters two and three, I used $3m^2$ semi-natural enclosures. These arenas are the same size as those used by other wild mouse researchers, and these semi-natural enclosures allow for the establishment of nesting areas and social hierarchies (Slade et al., 2014). Establishing competitiveness and mating success for males carrying a *t-Sry* construct in increasingly complex and naturalistic environments will be critical in determining the overall success of a genetic eradication approach for invasive mice on islands.

Gene Drive Rodents for Conservation-Broader Considerations

While reproductive and male competition studies will be critical for assessing the utility of a gene drive approach, they are only part of the overall task of assessing the feasibility of a genetic technique for invasive rodent eradication on islands. Several ecological and island-specific population considerations would also need to be modeled and understood before trials could commence (Backus & Gross, 2016; Leitschuh et al., 2017). It is also likely that gaining social and public acceptance will be equally, if not more, critical than the technology development process (National Academies of Sciences, Engineering and Medicine, 2016). Chapter four uses scenario analysis to address the eradication of rodents on inhabited and

uninhabited islands by specifically comparing the traditional approach of using rodenticides with approaches using sex-biasing gene drives. As gene drives are a new technology and still in development, input from the relevant public(s) and regulatory authorities will be very important moving forward and this input is also likely to lead to additional interesting and important questions that genetic construct developers will need to address.

Dissertation Program Overview

In the Fall of 2013, I was awarded a fellowship from the National Science Foundation, Integrated Graduate Education and Research Traineeship (IGERT) grant awarded to NC State University's Genetic Engineering and Society Center. As an IGERT fellow, I was part of a six-member cohort tasked with exploring what genetic engineering has to offer for combating the problem of invasive rodents on islands. Working as an interdisciplinary team of graduate students, we decided to create a website that explores the use and impacts that gene drive rodents may have on wild populations, ecosystems, and human society (https://research.ncsu.edu/ges/igert/student-research/island- mice-conserving-island-biodiversity; Backus et al., 2014). Stemming from the Genetic Engineering and Societies Center's engagements with the non-governmental organization Island Conservation, the Genetic Biocontrol of Invasive Rodents Program (GBIRd) was developed (http://www.geneticbiocontrol.org/). GBIRd is exploring the feasibility of using a gene-drive mouse for island population extirpation. As part of this organization, I have been given the unique opportunity as a graduate student to see how partnerships are formed and how government, academic, and non-governmental institutions can work together towards a common goal.

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Chapter 2

Towards a genetic approach to invasive rodent eradications: Assessing reproductive competitiveness between wild and laboratory mice

Research in this chapter is in press with the following journal:

Serr, M. Heard, N., Godwin, J., (2018). Towards a genetic approach to invasive rodent eradications: Assessing reproductive competitiveness between wild and laboratory mice. *Island Invasives Conference Proceedings,* International Union for the Conservation of Nature, Gland, Switzerland.

Abstract

House mice are significant invasive pests, particularly on islands without native mammalian predators. As part of a multi-institutional project aimed at suppressing invasive mouse populations on islands, we aim to create heavily male-biased sex ratios with the goal of causing the populations to crash. Effective implementation of this approach will depend on engineered F1 wild-lab males being effective secondary invaders that can mate successfully. As a first step in assessing this possibility, we are characterizing genetic and behavioral differences between *Mus musculus* strains in terms of mating and fecundity using wild house mice derived from an invasive population on the Farallon Islands (MmF), a laboratory strain C57BL/6/129 (t^{w2}), and F1 wild-lab offspring. Mice with the '*t*-allele' (t^{w2}) have a naturally occurring gene drive system. To assess fertility in F1 wild-lab crosses, t^{w2} males were paired with wild-derived females from the Farallon Islands (MmF). Results of these matings indicate litter sizes are comparable but that weaned pup and adult wild-lab mice are heavier in mass. Next, we initiated tests of male competitiveness using larger (three m^2) enclosures with enrichment. We introduced both an MmF and a t^{w2}-bearing male to two MmF females to assess mating outcomes. Preliminary results of these experiments show none of the offspring carried the *t*-allele. However, performing the same experiment with F1 wild-lab males instead of a full lab background resulted in 70% of offspring carrying the t^{w2} allele. This indicates that F1 wild-lab males may be able to successfully compete and secondarily invade. It will be important in subsequent experiments to determine what characteristics contribute to secondary invasion success. More generally a better understanding of characteristics contributing to overall success in increasingly complex and naturalistic environments will be critical in determining the potential of a gene-drive based eradication approach for invasive mice on islands.

Introduction

Invasive rodents are a key biodiversity threat for the majority of the world's islands and eradication campaigns are often employed to prevent loss of island endemics (Howald et al., 2007; Campbell et al., 2015). These eradications employ rodenticides and have been successful in eliminating invasive rodents from over 400 islands (Island Conservation, 2017). Rodenticides, however, have a higher failure rate with mice as opposed to rats (MacKay et al., 2007) and their use on inhabited islands presents severe logistical challenges. Additionally, rodenticides are not species-specific and present animal welfare concerns (Campbell et al., 2015). These challenges have created a compelling need for alternative approaches to rodent eradication.

One potentially promising approach to eliminating invasive mice from islands would be to bias offspring sex ratios by genetically engineering mice that produce only one sex of offspring. Pairing this approach with a genetic drive mechanism to spread this trait in an invasive mouse population would be critical. Key first steps are to understand the processes of reproductive competitiveness and the capability of an introduced mouse to introgress into established island populations, a process we are terming 'secondary invasion'. The phenomenon of secondary invasions and multiple introductions has been documented in invasive brown anole (*Anolis sagrei*) populations with evidence that secondary invasions may be frequent and can add genetic variation to existing invasive populations (Kolbe et al., 2004). This secondary invader phenomenon in house mice, however, is less well understood and genetic evidence suggests variation in how this occurs across islands. Some studies suggest that secondary invaders may be frequent (Berry et al., 1991; Bonhomme & Searle, 2012) while others suggest instead only single primary invasions (Hardouin et al., 2010; Gabriel et al., 2015). For rodent eradications these

secondary invaders would be carrying the gene drive and spread of this construct through the population would be necessary for this approach to be effective.

The development of the CRISPR/cas9 genome editing technology has recently revolutionized genetic engineering capabilities (Barrangou & Doudna, 2016). This has increased interest in genetic pest management approaches first conceptualized by Burt (2003) and built upon by other authors more recently (Sinkins & Gould, 2006; Esvelt et al., 2014). Many of these approaches center on gene drives, systems in which a genetic construct producing a desired phenotype (e.g., sex ratio manipulation, sterility) is preferentially inherited by offspring. These are considered 'selfish' genetic elements because the majority of offspring will inherit the genetic construct and it therefore could spread quickly through a population (Lyttle, 1991). In mice, a naturally occurring gene drive is found on chromosome 17 and is termed the *t*-allele (Silver & Buck, 1993). The *t*-allele bearing sperm impact the motility of non-t bearing sperm and this leads to an inheritance rate of greater than 90% for the *t*-allele (Bauer et al., 2005; Baker, 2008). Homozygosity of the *t*-allele (*t/t*) is typically lethal, but this is not true of the t^{w2}, although homozygosity does cause sterility (Dunn & Levene, 1961).

A gene drive based approach to eradication could use either a naturally occurring drive or a synthetic drive based on CRISPR/Cas9 and functional drives with this technique have now been demonstrated in mosquitoes, flies, and yeast (Harris et al., 2012; Dicarlo et al., 2015; Gantz & Bier, 2015); see also early contributions by Craig et al., 1960 and Hamilton 1967. Theoretically, by biasing offspring sex ratios heavily towards males, reproduction could be impaired, and populations reduced. One way this could be done would be to use the *Sry* gene. The *Sry* gene is the key male determining factor in mammals and is sufficient to start the cascade of events leading to male development (Hacker et al., 1995). Placing the *Sry* into an autosome

induces development that is phenotypically male in mice that are genotypically XX (Koopman et al., 1991). Inserting *Sry* into a naturally-occurring gene drive such as the *t*-allele or a synthetic drive based on CRISPR/Cas9 should create the potential for reduction of an invasive mouse population by reducing and ultimately potentially eliminating production of fertile females (Figure 1; Backus & Gross, 2016; Piaggio et al., 2017; Prowse et al., 2017). A synthetic gene drive using CRISPR/Cas9 could theoretically be employed in a similar way to ensure all offspring inherit a feminizing gene.

Regardless of the genetic mechanism employed, the reproductive competitiveness and relative fitness of gene drive carriers are likely to be important in determining the success of any genetic approach to reducing invasive mouse populations. Assessing reproductive competitiveness is the focus of this study. Since mice introduced with a gene drive mechanism would essentially be secondary invaders into an established invasive mouse population, it is important to better understand processes affecting introgression into established demes. Mice are social animals and dominant males will often hold and defend a territory (i.e. deme) that provides reproductive access to reproductive females while subordinate males do not (Bonhomme & Searle, 2012). How incoming mice are able to successfully integrate into island demes is not clear. If a gene drive approach were used, then the incoming males would need to compete with the resident island males for females. Competition and aggression tend to occur between male mice when there are limited territories (Gray & Hurst, 1998). Mouse populations living non-commensally on islands can instead exhibit an 'island syndrome' where they show important differences with commensal populations. These can include increases in body mass and, importantly in the context of this study, lower levels of aggression (Adler & Levins, 1994; Gray & Hurst, 1998; Cuthbert et al., 2016). In the 1980's, a study was conducted by capturing

house mice on the Orkney island of Eday (commensal) and releasing them onto the Isle of May, which was uninhabited by humans but had an established population of non-commensal wild house mice (Berry et al., 1991). This study followed the spread of genetic markers unique to Eday and found that these alleles moved quickly through the Isle of May population (Berry et al., 1991; Jones et al., 1995). Differences in aggression may relate to whether the mice are living commensally or not, with evidence indicating that commensalism and perhaps increased density favors more aggressive individuals (Berry et al., 1991; Gray & Hurst, 1998). Overall, the limited studies to date have strongly suggested that island mice may not be as competitive as their mainland/commensal counterparts (Mackintosh, 1981; Berry et al., 1991; Gray & Hurst, 1998).

Secondary invader success may also depend on female mate choice (Jones et al., 1995). In terms of female mate choice, there is evidence that females prefer the scent of foreign males and are more likely to mate with unrelated males (Roberts & Gosling, 2003; Frynta et al., 2010). Importantly, however, there is also evidence of female choice favoring non-*t* haplotype carrier males or males carrying a different *t*-haplotype variant (Lenington et al., 1994; Manser et al. 2015; Sutter & Lindholm, 2016). The relative fitness of gene drive carriers will be a critical determinant of effectiveness of this approach. Fitness costs have been documented with other forms of the *t*-allele (Carroll et al., 2004; Lindholm et al., 2013), but have not been examined for the t^{w2} variant to our knowledge. Information about the *t*-allele presence on islands and modelling of population dynamics would help us further understand the transmission of the *t-Sry* gene drive in island mouse populations (Backus & Gross, 2016).

Central Questions:

A critical aspect of exploring gene drive eradication techniques for island rodents is that the gene drive originates in a mouse strain with a standard laboratory background that is amenable to manipulation. Laboratory mice, however, have been inbred and housed in non-hierarchical social conditions for generations (Morse, 2007; Fawcett & Rose, 2012) and they have also undergone both deliberate and inadvertent selection under these captive conditions (Fawcett & Rose, 2012). It is encouraging to note, however, that wild-type behavior can be restored quickly by backcrossing with wild-derived mice to create wild-lab crosses (Chalfin et al., 2014). The central goals of this study are to one i) confirm that a gene drive mechanism can be bred into a wild background and ii) assess whether key reproductive measures such as litter size, pup weight, and adult weight are impacted in F1 and F2 wild-lab mice. We also present preliminary findings regarding the success of laboratory and F1 wild-lab males in competitive mating situations.

Materials and Methods

These studies employed several different strains of mice. A primary laboratory strain is C57BL/6J referred to as (B6) mice. B6 mice are the most common strain of lab mice and are easily manipulated genetically (Silver, 1995). Compared to other laboratory strains B6 mice are considered more defensive and aggressive in response to perceived threats (Blanchard et al., 2009). A second strain was donated from the Threadgill lab at Texas A&M University. These mice are of a mixed C57BL/6J and a 129S1/SvlmJ (B6;129) background (hereafter referred to as "lab" strain) and carry the t^{w2} variant of the *t*-allele. The t^{w2} variant stems from a wild background but was brought into laboratory stocks in 1946 (Dunn & Morgan, 1953). These mice

are not transgenic (no *Sry* inserted) and so heterozygotes produced are either male or female. The t^{w2} allele is inherited by 95% of offspring in matings with a $t^{w2}/+$ sire (Kanavy & Serr, 2017). To maintain t^{w2} mice, B6 females are mated to males heterozygous for the t^{w2} allele (t/+). The wild-derived mice (MmF) we use are derived from wild progenitors captured on Southeast Farallon Island, which is part of the Farallon National Wildlife Refuge, located about 30 miles off the coast of California near San Francisco (USFWS, 2013). Invasive mice are the only terrestrial mammals on the island currently (Schoenherr et al., 1999; USFWS, 2013). These mice show annual cyclic population variation with peak densities in late summer and early fall. MmF mice do not carry the *t*-allele (Threadgill pers. comm. 2013). Some of the highest mouse densities ever recorded in non-commensal habitats are seen on Southeast Farallon Island at over 1300/hectare (490/acre) (Seamons, 2013; USFWS, 2013;). Their diet consists primarily of invertebrates (Jones et al., 2006). The Farallons mice pose direct threats to an endemic invertebrate and indirect threats to native seabirds. The USFWS plans for a future mouse eradication with rodenticide (USFWS, 2013). We established a colony of wild-derived Farallons mice (MmF) at NCSU in 2013 and they are now 8th generation derived from the wild. These Farallon mice serve as the 'island mouse' model being used to form demes for testing the ability of secondary invaders to establish and mate successfully.

All experiments were conducted under an approved Institutional Animal Care and Use Committee protocol at North Carolina State University between 2015-2017. Mice were maintained in a temperature-controlled greenhouse with natural lighting and conditions suitable for reproduction year round. Animals were fed *Ad-libitum* with 5058 LabDiet® and daily health and welfare checks were performed. To test if mating between wild-derived MmF females and laboratory males occurred pairs of lab males with wild-derived MmF females were created and

housed in 29cm wide x 40cm long x 19cm high standard laboratory cages. Each cage contained aspen bedding, natural cotton, a 15 cm PVC tube and black oil sunflower seeds for enrichment. Mice were housed in this manner with weekly cage changes. To minimize disturbance, mice were transferred over to a clean cage using a 15 cm PVC pipe whenever possible. Pups were weaned at the mouse standard of 21 days +/- 3 days (Silver, 1995) and the litter size, sex and weight of the pups in grams were recorded. In addition, an ear punch or tail snip was taken for genotyping. Pups were then weighed as adults and their weight in grams was collected for nulliparous individuals between the ages of 70-140 days.

Tests of male competition were conducted in semi-natural enclosures. The size of these 'arenas' is $3m^2$, closely approximating the size of those used by Slade and co-workers (2014). To allow for formation of hierarchies and nesting, we added enrichment and complexity in the form of sand, bricks, plastic blocks ('Legos') supporting multilevel clear Plexiglass structures, galvanized wire mesh (1.25 x 1.25 cm mesh size), cardboard boxes and cardboard egg cartons, and PVC pipes for environmental complexity. For trials, all mice were placed into the arena at the same time. Males were either weight matched to within 1 gram (~5% of body weight) or age matched within 8-10 weeks. All mice used in the arenas were nulliparous and sexually mature. Colored ear tags as well as Clairol 'Just For Men' Black Hair dye® was used to identify males. Trials included combinations of MmF and t^{w2} males as well as MmF and F1 wild-lab males. At the start of each trial, both males and two non-related MmF females were placed into the arena and filmed for one hour. During this hour, we counted the number of bouts, chases and attempts to copulate, or time in proximity with females, as a means of assessing dominance. Animal welfare checks and monitoring for pups were performed daily. Any pups born in the enclosures were removed and a tissue sample was collected for genotyping.

To confirm the presence of the t^{w2} haplotype, we used a modified protocol where we amplified a portion the Hba-ps4 (alpha-globin pseudogene-4) locus (Schimenti & Hammer, 1990). The procedure uses a 'dirty' DNA extraction developed by one of our collaborators at Texas A&M University (Kanavy pers. comm. 2016). Tissue is collected and either a 2-3mm tail snip or a 2mm ear punch is used. The 'dirty' DNA extraction buffer contains (50µl 5 M NaOH, 4µl 0.5M EDTA, and 10mL sterile water). 100ul of extraction buffer is then added to the tissue sample and incubated at 95°C for 20 minutes. After vortexing and cooling 5µl of 1 M HEPES is added. The sample is then centrifuged at 6,000g for five minutes and 40µl of DNA is extracted from the top. DNA electrophoresis of PCR products shows a distinct band at 198bp for wildtype mice (+/+) while t^{w2} homozygotes (t/t) display a band at 214bp and heterozygotes (t/+) show the presence of both bands.

Statistical analyses were conducted using JMP® Pro 12.2.0 (SAS) where 1-way ANOVAS were used for adult weights and litter sizes. A mixed model ANOVA with the fixed effect of litter size was used to separate litter size from pup weight to compare pup weights. Following significant results in ANOVAs, mixed-model post-hoc analyses including orthogonal contrasts and Tukey's HSD tests were used to identify group differences. Litter sizes and weights are presented as mean ± SEM.

Results

Adult weights were taken for males and females. Sample sizes for males were as follows: B6 (33), t^{w2} (24), MmF (53), F1 (21), and F2 (22). For females sample sizes were: B6 (19), t^{w2} (25), MmF (44) and F1, (23). The average day of age that adult male weights were measured at was the following: B6=80.43± 21.95, t^{w2} = 90.43±27.65, MmF 92.63±34.90, F1 93.03±19.46,

and F2 89.48±28.27. Similarly, for females the average day of age that the adult weight was taken was: B6 91.24±28.99, t^{w2} 88.66±24.09, MmF 89.20±36.14, F1 82.25±38.15. Adult weights varied by strain and sex, $F_{8,257}$=28.35, p<0.0001. In addition, t^{w2} carrying males (t^{w2}, F1, F2) were larger than MmF males, F=58.00, p<0.0001. Similarly, t^{w2} carrying females (t^{w2} and F1) were larger than MmF females, F=7.75, p=0.0058 (Figure 2). Due to space restrictions for husbandry, not enough F2 adult females have been reared to allow calculation of a meaningful average for this group.

While litter size varied across strains $F_{5,141}$=4.59, p<0.0007, MmF, F1 and F2 wild-lab mice had litter sizes that were comparable (Figure 3). Sample sizes for litter size were as follows: B6 (27), t^{w2} (20), MmF (45), MmF/B6 (19), F1 (21), and F2 (20). There were no differences detected in the sex ratios for pups born, nor in the time of gestation (Data not shown).

Weaning weight was measured with a mixed model ANOVA with litter size being a fixed effect. The samples are as follows: B6 (18), t^{w2} (14), MmF (44), MmF/B6 (20), F1(13), and F2 (20). Pup weaning weight, $F_{133,383}$ =13.922, p=0.0001 reveals significant differences in pup weights across strains and the highest weaning weights were found in wild-lab F2 and F1's respectively (Figure 4). Highest mean weights at weaning are for F1 10.46± 0.40 and F2 9.82± 0.33 grams.

In the arenas, preliminary trials of male competition between t^{w2} males (laboratory strain) and MmF males revealed no t^{w2} transmission based on genotyping (3 trials with 35 pups total). The t^{w2} male initially appeared behaviorally dominant. He pursued females and chased the MmF male away, but on subsequent days was subordinate and tended to stay on top of the feeder out of view from the MmF male. Preliminary trials with MmF males and F1 wild-lab males (8 trials, 47 pups) revealed strongly contrasting results and a 70% transmission rate of the t^{w2} allele. Here

five of the eight litters did carry the t^{w2} with 31 of 33 pups from these litters confirmed. The F1 wild-lab males appeared to be behaviorally dominant throughout the trial in the same five trials where t^{w2} pups were produced. Dominance was again based on initiation of chasing or fighting with the MmF male and by time spent pursuing or mating with females. When subordination did occur, the subordinate males appeared to place themselves so as not to be visible to the dominant individual. Behavioral results are ongoing and were beyond the scope of this manuscript.

Discussion

Relative fitness of gene drive carriers is likely to be critical in determining the success of this approach (Burt, 2003; Manser et al. 2015; Backus & Gross 2016). Carriers of gene drive constructs would need to be successful in reproduction and reproductive competition if a genetic approach to invasive rodent eradication is to be effective. This work establishes some key initial conditions for this success. First, lab mice and wild mice can breed and produce viable litters. Second, while litters of the common lab background t^{w2} mice were smaller than those of wild-derived mice under the more naturalistic conditions used in this study, the F1 wild-lab litters were of comparable size to those having two wild-derived parents. Preliminary results also suggest F1 wild-lab males may have strong potential for reproductive success, a likely prerequisite for initial introgression of gene drive constructs into an island population.

This work established that wild-derived Farallon females will mate with laboratory males in standard cages and at similar frequencies to those seen in matings with wild-derived males (data not shown). This was an initial but critical step in assessing reproductive output across strains and in F1 wild-lab mice. Furthermore, results indicate that both F1 wild-lab and F2 wild-lab backcrossed mice have litter sizes that are not different statistically than those of Farallon

mice. This is important in terms of fitness and exploring the effectiveness of utilizing the *t-Sry* haplotype technique. It is also important to note that the reverse holds true, as wild-derived MmF males will mate with B6 and t^{w2} females in standard laboratory cages although sample sizes are not adequate for statistically meaningful comparisons. Results for pup weights indicate F1 and F2 wild-lab pups have the greatest weight at weaning and that this trend continues for adult males. Body size affects male competitiveness in mice (Cunningham et al., 2013; Ruff et al., 2017) with evidence suggesting that in semi-natural enclosures male mice of intermediate weight have the highest fitness (Ruff et al., 2017). Matching mice based on body size for our experiments helps rule out this confounding factor, but for a potential gene drive release it could be beneficial for the drive-bearing mice released to weigh more than their wild counterparts.

Preliminary results from experiments in our larger arenas examining competition suggested a surprising pattern. Arena trials between MmF and t^{w2} males suggest the wild-derived MmF males are dominant to pure laboratory strain males, preventing transmission of the t^{w2} allele. Interestingly, however, weight-matched F1 wild-lab males carrying the t^{w2} allele appear more competitive and behaviorally dominant to MmF males. Consistent with this observation, we find a 70% transmission rate of the t^{w2} allele in arena trials analyzed thus far. In addition, of the three trials where the F1 wild-lab male was not dominant MmF litter sizes were small with two of the three litters only having two pups each. This suggests that F1 wild-lab males are strong competitors and that females will mate with F1 wild-lab males even when both male types are present. It will be important to conduct further arena trials to assess this competitiveness with greater sample sizes and also assess the competitiveness of F2 wild-lab males. Other reproductive comparisons we are conducting include measuring testes weights. Testes weight is correlated to total sperm count in mice (Le Roy et al., 2001). Testes weight can also predict

dominance and mating success, as mice with higher testicular weight are more likely to initiate mating with females and attack behavior towards conspecific males (McKinney & Desjardin, 1973). Finally, nesting behavior and the temperature of nests will be important to examine across wild-derived, laboratory and F1 wild-lab mice as anecdotal observations suggest poor nest construction by laboratory mice. This could be important too because in cooler environments studies have indicated that nest building behavior, thermoregulation, and fitness are correlated (Bult & Lynch, 1997).

Our results suggest that F1 wild-lab males could be efficient secondary invaders. This would be generally consistent with other studies from island populations (Jones et al., 1995; Bonhomme & Searle, 2012). However, the situation may be different for females. Introduction of mice from a commensal population on the Isle of Eday to the Isle of May did not lead to the spread of mitochondrial DNA markers, which are maternally inherited. These results were in contrast to those for a Y-chromosome marker and suggested females were unable to secondarily establish while males did (Jones et al., 1995). Studies from other islands have corroborated these results in suggesting no integrations of new maternal haplotypes from later-arriving females (Searle et al., 2009; Gabriel et al., 2010; Jones & Searle, 2015). This apparent male-female asymmetry in secondary establishment ability, however, has not been experimentally tested. One approach to addressing this apparent asymmetry is having records of detailed behavior in more naturalistic arena settings. We have designed and implemented a Radio Frequency Identification (RFID) system for tracking mouse movements. RFID tracking allows collection of detailed behavioral records and works well with wild house mice (Weissbrod et al., 2013; Auclair et al., 2014). Behavioral measures include capturing time spent at nest boxes, running wheels, and food. With this information we can assess the number of visits, the timing of visits, the number

of interactions, and time in social contact with one another (König et al., 2015; Lopes et al., 2016).

A second approach is to test the ability of different strains to establish dominance in a standard test termed resident-intruder paradigm. A previous study used this approach to compare competitive behavior in house mice from the Isle of Eday and the mainland, finding the island mice were significantly less aggressive (Gray & Hurst, 1998). Expanding trials to increasingly complex naturalistic experimental arenas should give insight into the relative abilities of male and female mainland mice to secondarily invade and therefore genetically introgress into an island population.

Other factors that could influence the potential success of an eradication effort include mate-choice and tolerance of island conditions. Mate-choice factors known for mice include odorant cues such as urinary proteins and ultrasonic vocalizations (Hurst & Beynon, 2004; Blanchard et al., 2009; Musolf et al., 2010). Island conditions and climate in particular could be important influences on the success of introduced mice (Berry, 1992). The island syndrome for rodents predicts increased body mass and decreased aggression (Adler & Levins, 1994; Gray & Hurst, 1998; Cuthbert et al., 2016). In addition, the island syndrome in rodents is often associated with high population densities, increased reproductive output, and increased survival rates on islands (Adler, & Levins, 1994). Mice are able to adapt to new conditions and islands (Anderson, 1978; Bronson & Pryor; 1983) and this adaptation could be critical for fitness, although any construct would presumably be introgressed into an island genetic background relatively quickly as it spread. The population genetic structure of the mice already present on an island would be critical for a synthetic gene drive, but other factors including the rate of inbreeding, ratio of reproductive males to females, and age structure of the mouse population(s)

might also prove important. These are also likely to impact spread of either a synthetic or natural drive like the *t*-haplotype considered here. In regions with seasonality and temperature variations, mouse populations often undergo a 'boom and bust' cycle, as seen in the Farallon Islands, where the populations can erupt only to die off with changes in temperature. The timing of release of secondary invaders will likely be important in these situations (Singleton et al., 2005; USFWS, 2013; Backus & Gross, 2016). Both natural and sexual selection could influence the number of drive carrier mice that would be required for eradication success. A study by Backus & Gross (2016) modelling the *Sry*/t^{w2} gene drive found that the relative fitness of the mice carrying the gene drive determined whether multiple releases would be required. Similarly, Prowse and co-workers (2017) modeled synthetic gene drives and found that a sex reversing drive would require multiple releases to achieve eradication success.

The concept of reducing invasive mouse populations through release of genetically-modified mice is still in the early stages of development. Many key issues will need to be addressed to determine whether this is a feasible approach. We have shown that an island-derived wild strain will mate with t^{w2}-carrying laboratory males and produce comparable litter sizes to those of wild-wild matings. Promisingly, we also see that pup-weaning weights are larger for F1 and F2 wild-lab mice and that F1 wild-lab males may be stronger competitors in semi-natural enclosures. A key future step will be to scale up trials in arena size and environmental complexity. Larger enclosures could be utilized with greater numbers of mice to test whether a gene drive can spread under controlled and biosecure, but naturalistic conditions. Finally, beyond the technical issues discussed above, social license for any environmental releases would be crucial (National Academies of Sciences, Engineering and Medicine, 2016). As gene drives are a new technology still in development, input from the relevant publics and

regulatory authorities will be very important moving forward and this input is also likely to lead to additional interesting and important questions that developers will need to address.

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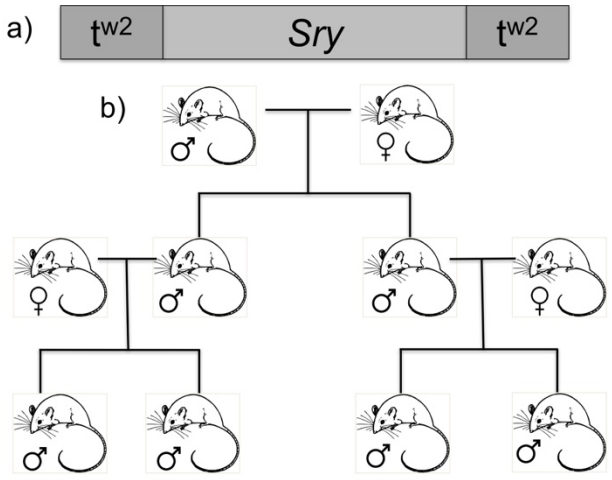

Figure 1. Construction and spread of the *t-Sry* gene drive **a)** Depiction of the *Sry* gene inserted into the t^{w2} gene drive **b)** a depiction of how the population would bias to be all male.

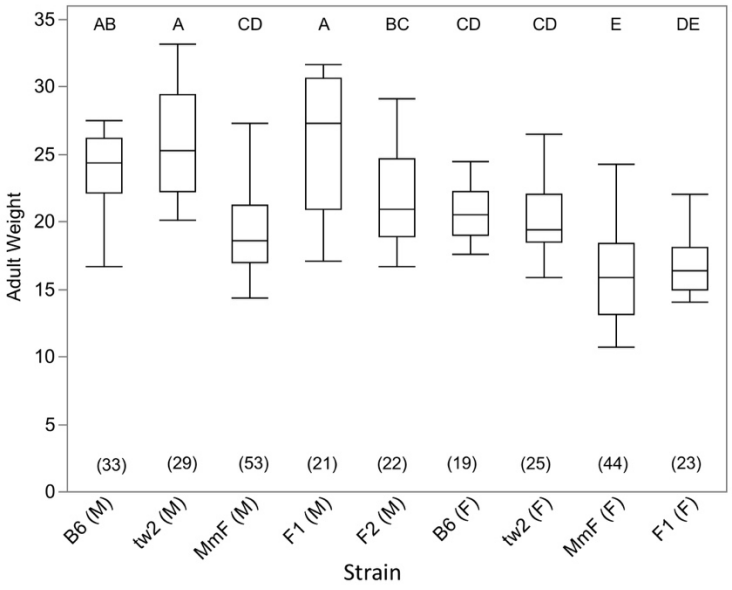

Figure 2. Adult mouse weight by strain and sex. 1-way ANOVA, $F_{8,257}$=28.35, p=0.0001. Tukey's HSD reveals significant differences in weights indicated by letters. Sample sizes are indicated in parentheses.

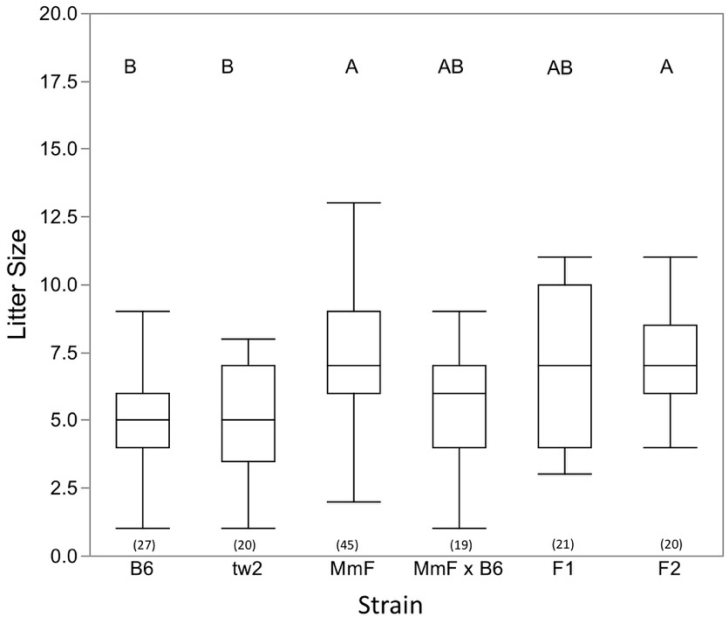

Figure 3. Litter sizes vary by mouse strain. 1-way ANOVA, $F_{5,141}$=4.59, p=0.0007 indicates significant differences in litter size across strains. Tukey's HSD reveals significant differences in weights indicated by letters. Sample sizes are indicated in parentheses.

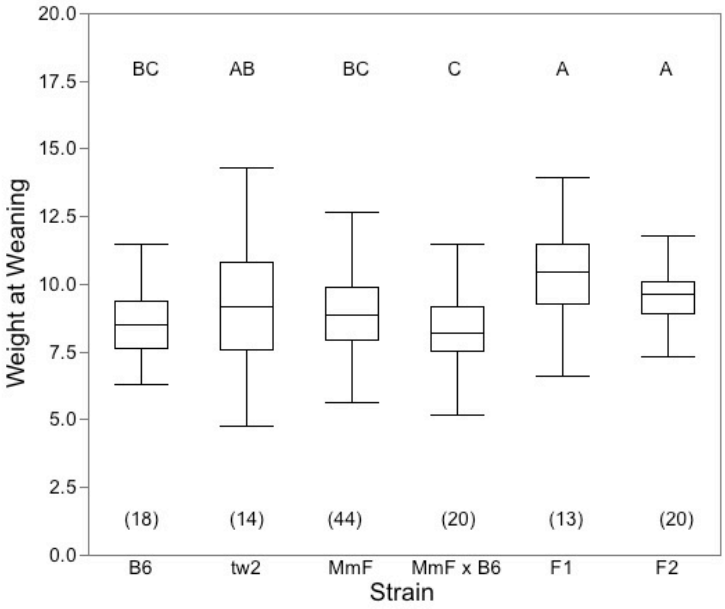

Figure 4. Weight at weaning varies by mouse strain. Weaning weight with fixed effect of litter size Mixed Model, strain $F_{5,119}$ =4.98, p=0.0004., litter size $F_{1,117}$=12.46, p=0.0006. Tukey's HSD and Orthogonal contrasts reveal significant differences in pup weights across strains, which is indicated by letters. Sample sizes are indicated in parentheses.

Chapter 3

Male mate competition studies to inform a meiotic drive approach in house mice (*Mus musculus*)

Abstract

As part of an international program GBIRd, we are exploring the removal of invasive house mice on islands with a genetically-engineered version of a natural meiotic distorter that would create male biased-offspring allowing the local population to be extirpated. The success of this technique would rely on genetically engineered males that carry the Y-linked gene within a meiotic distorter *t*-haplotype (t^{w2} variant) mating successfully with females of the targeted island population. To inform this aim we have been testing reproductive and behavioral interactions between wild-derived and laboratory or wild-lab (hybrid) mice. To test male competition among wild-derived and laboratory mice, we constructed ($3m^2$) large arenas and allowed both a wild-derived and a non-engineered, but t^{w2}-bearing laboratory male access to two wild-derived females. Results of these experiments showed that none of the offspring carried the *t*-haplotype. However, performing the same experiment with hybrid F1 and F2 backcrossed males resulted in > 80% of offspring carrying the t^{w2}. In addition, during the first hour after introduction we assessed behavioral interactions. These experiments determined that wild-derived males are more prone to retreating from a fight when hybrid males are present and that hybrid males copulate more often with females. Further reproductive characterizations showed that F1 wild-lab males have larger testes sizes relative to body weight and that mating order influences sperm competition and siring of offspring, while carrying of the t^{w2} did not influence likelihood of paternity. These results indicate that hybrid males may successfully compete against wild males on islands. Selecting for characteristics contributing to male mating success in increasingly complex and naturalistic environments will be critical in determining the overall success of a genetic eradication approach for invasive mice on islands.

Introduction

Invasive rodents pose a significant threat to native species on islands (Wanless et al., 2007; Bellard et al., 2016), most notably for seabirds but also for other fauna and flora (Towns et al., 2006; Fukami et al., 2006; Caut et al., 2008; Hilton & Cuthbert, 2010). Conservationists have demonstrated that eradicating invasive rodents from islands results in substantial conservation gains and increases seabird populations (Capizzi et al., 2014; Jones et al., 2016). The number of rodent eradications has increased with over 650 attempts on islands worldwide and a failure rate of less than 20% (Holmes et al., 2015). These eradication efforts have also grown in size to islands as large as South Georgia island (30,000ha) in the southern Atlantic ocean (Simberloff et al., 2018). The current method for invasive rodent eradication for islands larger than 5ha is rodenticides, spread aerially in bait form (Campbell et al., 2015). These rodenticides are anticoagulants with the most commonly used and effective being brodifacoum (Howald et al., 2007; Parkes et al., 2011). However, use of rodenticides has several drawbacks as these chemicals are not species-specific, can persist in the environment, and resistance can develop (Parkes et al., 2011; Masuda et al., 2015; Berny et al., 2018). There are also negative public perceptions regarding the use of rodenticides (Fitzgerald, 2009; Cowan & Warburton, 2011), which can delay and hamper eradication efforts (Novoa et al., 2018). Furthermore, the current aerial methods with rodenticides have only been used on uninhabited islands, as there are increased challenges and safety precautions when humans are present, who could potentially be exposed to rodenticides (Oppel et al., 2011; Glen et al., 2013; Campbell et al., 2015). Hence, while rodent eradications have increased in terms of the number and size of the islands where this approach is applied, a major challenge remains for many islands where eradication remains difficult or impossible. Indeed, an estimated 85% of islands where invasive rodents represent a

biodiversity threat are not suitable for rodenticide-based eradications (Campbell et al., *in press*). This challenge has prompted researchers to explore new rodent eradication technologies (Campbell et al., 2015).

One technology being explored is a genetic approach that creates a sex-biased population, causing population declines due to decreases in reproduction (Campbell et al., 2015; Leitschuh et al., 2017). As part of the Genetic Biocontrol of Invasive Rodents program (GBIRd, www.geneticbiocontrol.org), we are looking at several genetic strategies that could achieve eradication using synthetic gene drives and modifications to naturally-occurring selfish genetic elements that are preferentially inherited (Campbell et al., 2015 and *in press*). This study focused on the *t*-haplotype, a naturally-occurring and well-studied meiotic drive found in house mice (Silver, 1993). The *t*-haplotype influences sperm motility in males and works as a poison/antidote system. The presence of the *t*-haplotype decreases the motility of non-*t* bearing sperm and *t*-bearing sperm are transmitted at a higher rate, with some variants showing transmission distortion rates of over 90% (Bauer et al., 2005; Baker, 2008; Herrmann & Bauer, 2012). Because the *t*-haplotype affects only sperm, transmission from females is unaffected (Lyon, 2003). We are using a variant of the *t*-haplotype termed the t^{w2} that transmits at a rate greater than 90% and is homozygous sterile, unlike other variants that are homozygous lethal (Dunn & Levene, 1961; Lyon, 2003). To create a male sex-bias in offspring produced, the goal is to insert the normally Y-linked male determining gene *Sry* into the t^{w2}. The *Sry* gene is critical for male development (Gubbay et al., 1990). A previous study showed that the presence of the Sry gene initiated male development, even when it is inserted into the autosome of a female (XX) mouse (Koopman et al., 1990). The XX/*Sry* mice were sterile but anatomically and behaviorally indistinguishable from other males. Based on this *Sry* effect, efforts are underway to

insert the *Sry* gene into the *t*-haplotype (Kanavy, 2018). The *t-Sry* is a construct that could theoretically create heavily male biased populations, as *t-Sry* males will pass the construct down to their offspring, and XX/*Sry* individuals will be phenotypically male but sterile (Backus & Gross, 2016; Piaggio et al., 2017; Leitschuh et al., 2017).

A necessary requirement of a *t-Sry* or likely any genetic approach would be ensuring that genetically modified males are reproductively successful following release on an island. Specifically, it is critical to assess males from wild, lab, and wild-lab cross backgrounds (hereafter hybrid) mice in a mate competition context. House mice in wild and semi-natural populations are polyandrous with litters of multiple paternity (Potts et al., 1991; Dean et al., 2006; Firman & Simmons, 2008). In these settings, mice establish social structures known as demes and males compete for access to females (Latham & Mason, 2004; Bonhomme & Searle, 2012). Male mice are territorial, and fighting is common when male mice encounter stranger males (Novotny, 1990: Latham & Mason, 2004). Previous research showed that dominant males sire more offspring by preventing subordinate males from accessing females (Novotny et al., 1990). To address this question, we focused on mating laboratory mice that carry the *t*-haplotype with wild-derived mice whose progenitors are from an invasive island population. Our island wild-derived mice are from the Farallon islands off the coast of California, USA. Because the *t-Sry* is still under development and also because of increased biosecurity-based logistical challenges that would be associated with working with a transgenic mouse, we instead used laboratory mice that carry the t^{w2} and are being used in transgenesis efforts. Our previous study showed that island-derived Farallon mice will mate with t^{w2} bearing laboratory males and produce comparable litter sizes to those of wild-wild matings (Chapter 2 and Serr et al., *in press*). Importantly for reproductive competitiveness, we found that adult weights are greater for

t^{w2} bearing males than Farallon males (Figure 6). Knowing that wild-derived and laboratory mice do indeed mate successfully in standard laboratory housing was an important first step that allowed us to proceed to this study, which focuses on reproductive interactions in more naturalistic environments using larger semi-natural enclosures. The primary questions addressed in this study are can *t*-haplotype (t^{w2}) carrying lab strain males reproduce successfully when in competition with wild-derived males? If not, what degree of backcrossing would be necessary to generate a competitive male mouse carrying the *t*-haplotype? Finally, are there differences in behavioral and reproductive characteristics that correlate with variation in male success in a competitive mating context?

Material and Methods

We conducted experiments under approved Institutional Animal Care and Use Committee protocols at North Carolina State University between 2016-2018. All mice were maintained at a mean temperature of 21±3.9 ℃ (± SEM, range 11°C- 26°C) with a mean humidity of 30.83 ±9.1% (range 25%-65%) in a greenhouse with natural lighting. Animals were fed *ad libitum* with 5001 LabDiet® (Purina, Creedmoor NC) and daily health and welfare checks were performed along with environmental temperature monitoring.

a) Strains of mice

We used several different strains of mice in these experiments (Table 1). Our wild-derived mice (MmF) were from founders originally collected from Southeast Farallon Island, (37°41' N, 123°0' W) part of the Farallon National Wildlife Refuge, located approximately 30 miles off the coast of San Francisco California, USA (USFWS, 2013). The refuge is off-limits to the general public and mice on the island live non-commensally. We chose the Farallon mice as

a model island invasive mouse population due to the high densities occurring there (up to ~1300/ha) (USFWS, 2013). These mice present a conservation threat and the United States Fish and Wildlife Service is planning for eradication of this population with rodenticide (USFWS, 2013).The progenitors were collected in the fall of 2013 and at the time of experiments the mice utilized were seven to eight generations removed from the wild. The Farallon mice do not carry the *t*-haplotype.

The laboratory strain of mice utilized in our experiments that carry the t^{w2} are on a mixed background C57BL/6J and 129S1/SvlmJ (B6:129). This strain was donated by Dr. David Threadgill at Texas A&M University and has since been maintained in our colony by breeding the t^{w2} bearing males with C57BL/6J females. The mice that carry the t^{w2} variant in these studies are not transgenic and no *Sry* has been inserted, hence they produce both male and female offspring. We used these mice as this is the genetic background and the *t*-haplotype variant that ongoing efforts to develop a transgenic, male-biasing construct are being conducted in. In the Threadgill Lab the t^{w2}-haplotype is inherited at a rate of 94% (Kanavy, 2018). Laboratory mice used in experiments were from the second and third generation in our colony and hereafter referred to as t^{w2}.

To generate an F1 hybrid cross we mated a t^{w2} laboratory male with a MmF female, resulting in a 50% wild background. We then performed a backcross to create what we termed an F2 backcrossed mouse that was an F1 male mated with a MmF female, resulting in a 75% wild background (Figure 1).

Table 1. Mouse strains utilized for experiments

MmF	Wild-derived island mice from the Farallon islands. 6-8 generations from the wild
t^{w2}	Mice carrying the t^{w2}-haplotype (B6/129 background) *Not transgenic*
F1 Hybrid	MmF/t^{w2} Mice with a 50% wild background Generated by mating a Farallon female with a t^{w2} male
F2 Backcross	Mice with a 75% wild background Generated by mating Farallon female backcrossed with a F1 hybrid male

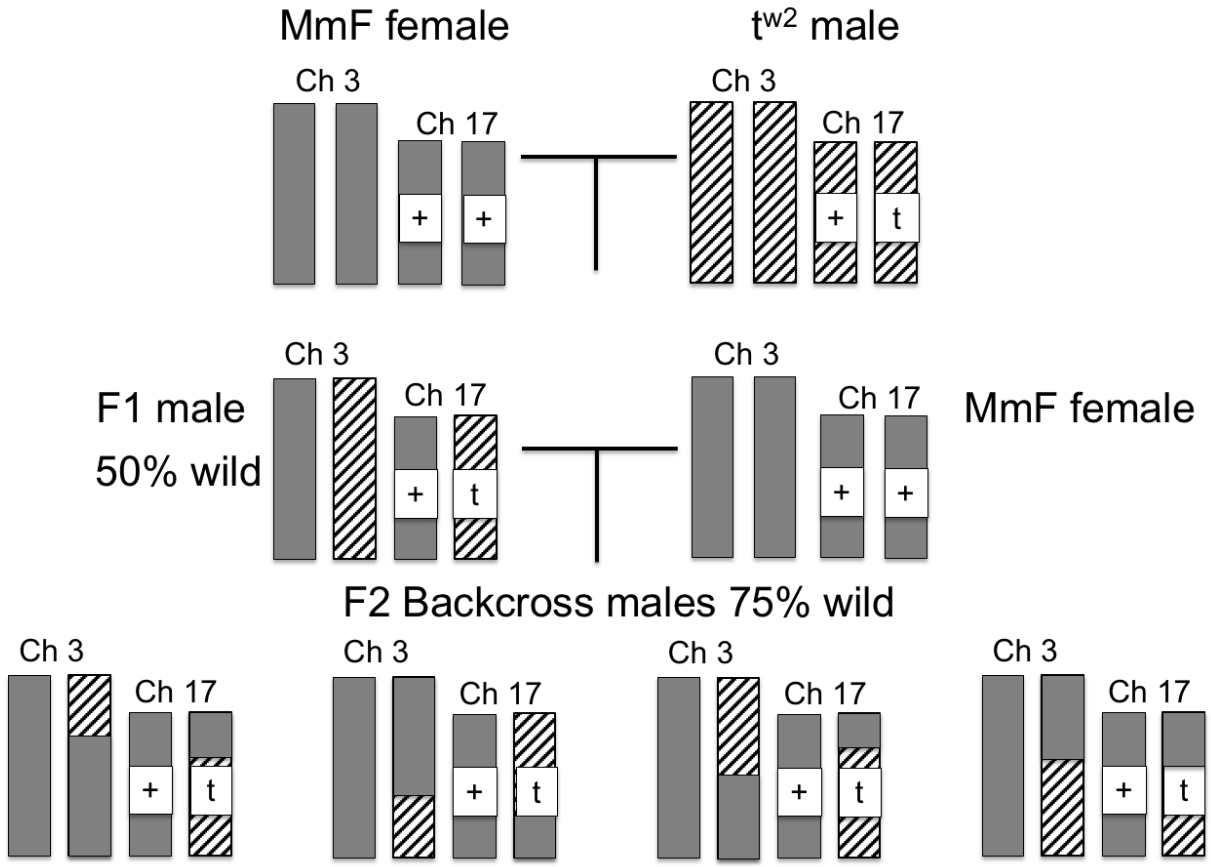

Figure 1. Chromosomes depicting the F1 cross and an F2 backcrosses. The t^{w2} is denoted with a t and wildtype with a +. An F2 backcross is generated by mating an F1 male with a MmF female.

b) Transmission Ratio Distortion

To confirm the presence of the t^{w2}, we collected a 2-3mm tail snip or a 2mm ear punch and then extracted the DNA with a 'dirty' DNA extraction buffer which contained (50µl 5 M NaOH, 4µl 0.5M EDTA, and 10mL sterile water). We used a protocol adapted from Schimenti and Hammer (1990) that identified the *t*-haplotype through PCR genotyping of the Hba-4ps locus. For a more detailed description, see Kanavy (2018). Mice that are wild-type (without the *t*-haplotype) have one band at 198 base pairs (bp). Mice with the *t*-haplotype contain a 16bp insert at this locus and have a second band resulting in a second 214 base pair fragment. Mice homozygous for the *t*-haplotype were not utilized in experiments but generate one band at the 214 bp fragment size.

c) Male Competition and Reproductive Success

Prior to experiments, we maintained mice in standard laboratory cages (29cm wide x 40cm long x 19cm high) until they were ready to be placed into competitive enclosures. We assessed male competition in semi-natural $3m^2$ 'arenas'. These arenas are the same size as those used by Slade et al. (2014) and allow for the formation of nesting areas and social hierarchies. To prevent disruption of existing social structures and increased aggression, we placed all individuals into the arena at the same time. All individuals were sexually mature, but under 18 weeks of age and nulliparous. Each arena group consisted of two Farallon females from the same litter, an unrelated Farallon male and an 'opponent' male (t^{w2}, F1 or F2). Males were either age-matched within one week or weight-matched to within 1g (~5% of body weight) depending on the experimental trial.

d) Factors Affecting Male Reproductive Competitiveness

About two days before being introduction into the arena, we ear tagged mice with different colored ear tags (Stoelting, Wood Dale Illinois). Males were also marked with Clairol 'Just for Men' Black Hair Dye on the right or left shoulder. At the start of the trial, all individuals (two MmF females, one MmF male, and one opponent male) were placed in at the same time. As noted above, opponent males were either a t^{w2} lab, F1 or F2 male. We video recorded and had two observers observe the first hour of initial interactions, one recorded all the male interactions, and the other female interactions. Our behavioral assessments followed Hurst et al (1996) and scored the following behaviors: approaches, retreats, investigations, fights, chases, pursuits and copulations. Briefly, approaches were encounters where males moved within 3cm of the other males while retreats were the active backing and turning away from the other mouse. Investigations involved sniffing a part of the other mouse and or the air. Fights involved mutual 'wrestling' or using forepaws to 'scrabble' as well as actively biting the other male (Hurst et al., 1996). We modified Hurst's definition of pursuit to only include instances when males actively went after female mice and used the term chase for when males went after other males. We also added copulations for when there was visible mounting of males on females that involved thrusting. As an ethical note based on Hurst et al. 1996, we predetermined that if there were more than ten fights in an hour or if there was visible wounding we would remove that individual from the enclosure. Although this did not occur for any trials. We observed the first hour of interactions and thereafter checked every three hours for signs of wounding or aggression until 12 hours after placement. Daily animal welfare checks occurred after the first day. Of the 27 arena trials conducted, there were five cases where one of the males was found dead several days, up to a week later. These males had no obvious signs of injury or major trauma. In six

cases, males were euthanized due to injuries. All efforts were made to limit aggressive interactions, but not all actions occurred within the first day or week nor could they be predicted based on previous trials and observations.

e) t-haplotype transmission in Arenas

We monitored for pups daily. Any pups born in the enclosures were accounted for, removed, and euthanized upon birth. Preliminary experiments had shown that early pup mortality loss was high and so waiting for pups to be of weaning age was not possible. In some cases litters were born at the same time and co-housed making the determination of which Farallon female gave birth not possible, but this was noted. For each pup, we took a tail tissue sample for t^{w2} transmission screening, utilizing the method described above.

f) Reproductive System Differences: See Supplementary Material

g) Statistical Analyses

We conducted statistical analyses in JMP$^{(R)}$ Pro 13.2.0 (SAS, Cary, NC) where a chi-square test was used for transmission rate comparisons. 1-way and 2-way ANOVAs were calculated for arena transmission rates followed by post-hoc Tukey's Honest Significant Difference (HSD) for comparisons where significant differences were present. We used a binomial test to assess the likelihood that there was paternity by the Farallon male for mixed litters and the number of individuals without the *t*-haplotype within the litter. We used One-way ANOVAs to compare behaviors between MmF males and opponent males.

Results and Discussion

Transmission Ratio Distortion

Transmission rates for the t^{w2} are consistent with other findings in having 94% or higher inheritance (Dunn & Morgan, 1953; Kanavy, 2018). For our laboratory background B6:129 mice, we found 97.64% transmission ratio distortion (207/212). Similarly, for our F1 hybrid mice we had 96.69% (234/242) and for our F2 backcrosses, we observed 96.15% (125/130) (Table 2). As reported in previous studies with the *t*-haplotype (Dunn & Levene, 1961; Lyon, 2003), we also found that when the female carries the t^{w2}, there were normal Mendelian rates of transmission 63/114 (55.26%), which is not significantly different than the expected 50% with a binomial distribution (p=0.26).

Table 2. Experimental crosses to generate test groups and measure *t*-haplotype transmission ratio distortion.

Strains of Mice	**Strain of pups produced**	**Percent of pups born with *t*-haplotype**
C57BL/6 female x C57BL/6/129 (t^{w2}) male	t^{w2} maintained on a laboratory C57BL/6/129 background	207/212=97.64%
Farallon female x t^{w2} male	F1 (50% wild derived)	234/242=96.69%
C57BL/6/129 (t^{w2}) female x Farallon male	F1 reverse (Half carry t^{w2})	63/114=55.26%
Farallon female x F1 male	F2 Backcross (75% wild derived)	125/130=96.15%
Total	**Overall transmission when male carries t^{w2}**	**566/584=96.92%**

The total for all samples where the male carried the t^{w2} was 566/584 (96.92%). This ~97% transmission rate is consistent with the 94.3% rate observed in lab mice with the t^{w2}

haplotype, but actually slightly and significantly higher (96.92%, n=584 in this study vs. 94.3% n=2349 in Kanavy, 2018; Chi-square= 6.546, p=0.011). These results indicate that transmission rates are not compromised in at least the wild-derived background tested here and may in fact even be higher in wild-derived mice when compared to standard laboratory mice. Confirming strong transmission ratio distortion in matings with wild-background females is important from an applied perspective as this would be critical for the effectiveness of a *t-Sry* mouse being introduced for population control.

Male Competition and Reproductive Success

Male competition trials showed that when the opponent was a t^{w2} lab male, there was zero percent transmission of the *t*-haplotype. Of the ten trials, five resulted in litters, but none of the 59 pups carried the *t*-haplotype. This does not appear to be due to an inability of purely lab-strain t^{w2} carrying males to produce pups since breeding tests in standard laboratory cages showed that t^{w2} males do not differ in their time to produce litters with Farallon females, compared to Farallon males (Chapter 2 and Serr et al., *in press*). The lack of success for t^{w2} males in the competitive arena setting therefore suggests a lack of competitive ability and not a reproductive difference. Interestingly, when the opponent male was a F1 hybrid male (eleven trials that all produced litters) there was 81.43% transmission of the *t*-haplotype (Figure 2). This was a surprising result that contrasted sharply with the results of the MmF-t^{w2} trials where there was no transmission. This pattern was also observed when we tested F2 backcrossed males with MmF males. All eight trials resulted in litters and 91.84% of the pups carried the *t*-haplotype.

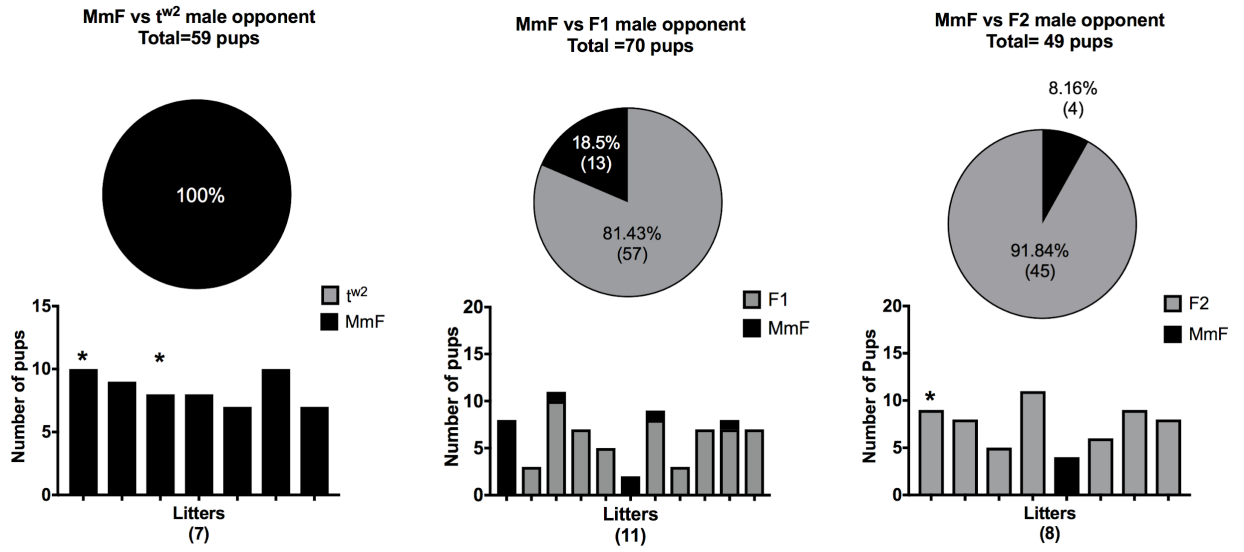

Figure 2. Male competition trial results: Pie charts depict the overall percentage of pups with either the wild-type (MmF) or the t^{w2}. Bar graphs depict the results of individual trials and the number of pups from each that carried the wild-type or the t^{w2}. * represents trials where litters were cohoused, and more than one female gave birth at the same time in the same trial.

As a conservative estimate, we assumed those offspring without the *t*-haplotype in trials were from Farallon males. As noted above, multiple paternity is a common phenomenon in the polyandrous house mouse (Potts et al., 1991; Dean et al., 2006: Firman & Simmons, 2008). However, it is also possible that the *t*-haplotype failed to transmit. For F1 opponent males there were three instances of mixed liters (Figure 2). The likelihood of one pup inheriting the wild-type allele in a litter sired by the F1 is 0.243, 0.212, and 0.194 respectively. For these cases this is indicative of non-transmission of the *t*-haplotype, though multiple paternity cannot be ruled out. For the F2 trials, one litter consisted of three wild-type offspring and no *t*-haplotype offspring, which would be extremely unlikely if the sire were a *t*-haplotype carrying male ($p < 0.001$).

Factors Affecting Male Reproductive Competitiveness

The finding that F1 and F2 hybrid males were stronger competitors than fully wild-derived MmF males in our arena trials was unexpected. This result led us to examine what might account for these differences in male success in reproductive competition. There are significant weight differences with t^{w2} carrying males being heavier than Farallon males (1-way ANOVA, $F_{3,104}$=17.924, p< 0.0001: Tukey's HSD, p<0.05; Figure 6). As t^{w2}-bearing males are larger than MmF males at the same age, we next conducted trials to separately assess potential effects of weight and age on male success. Testing revealed there was no significant effect of either age or weight on reproductive success but that type of opponent did have a significant effect (2-way ANOVA matched by age or weight $F_{2,2}$=0.002, p=0.999, opponent $F_{4,18}$=4.249, p=0.014 Figure 3). Taken together, these results indicate that strictly laboratory-derived males are not competitive with wild-derived Farallon males, but that F1 and F2 hybrid males are more effective competitors than Farallon males even when without a weight advantage.

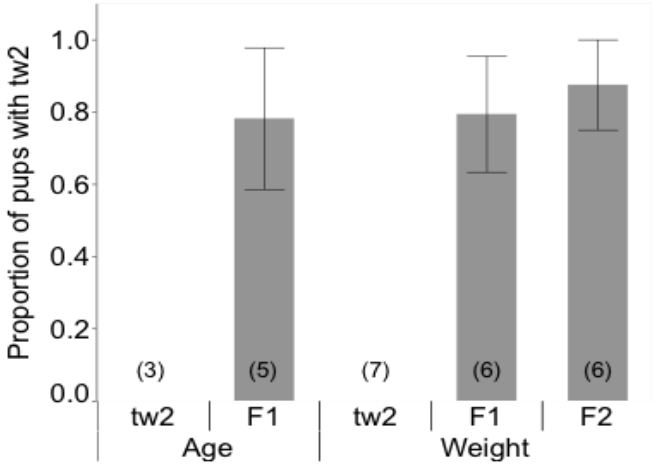

Figure 3. Proportion of offspring with t^{w2} matched by age or weight. Sample sizes are indicated in parentheses.

In further examining what might account for differences in male competitive success, we analyzed the behavior seen during the first hour following introduction. While video recording during the first hour was successful, it was sometimes difficult to determine which male initiated certain behaviors. Instead we relied on the observations taken from the two observers as they live scored the behaviors performed. Results of these observations indicate that the Farallon males retreated more often than opponent males (1-way ANOVA, $F_{2,24}$=4.820, p=0.017 Figure 4a). Related to retreats, there was no significant difference in the number of fights that occurred across opponent males (1-way ANOVA $F_{2,24}$=2.912, p=0.0738), but the latency until the first fight was shorter for both hybrid F1 and F2 backcrossed opponents than t^{w2} opponents (1-way ANOVA, $F_{2,24}$=4.753, p=0.025; t^{w2} > (F1=F2), Tukey's HSD; Figure 4b). Similarly, while the number of retreats increased for Farallon males when paired with a hybrid male relative to a t^{w2}, the number of chases by males did not differ by opponent or for the Farallon males (1-way ANOVA, $F_{2,24}$=2.010, p=0.162).

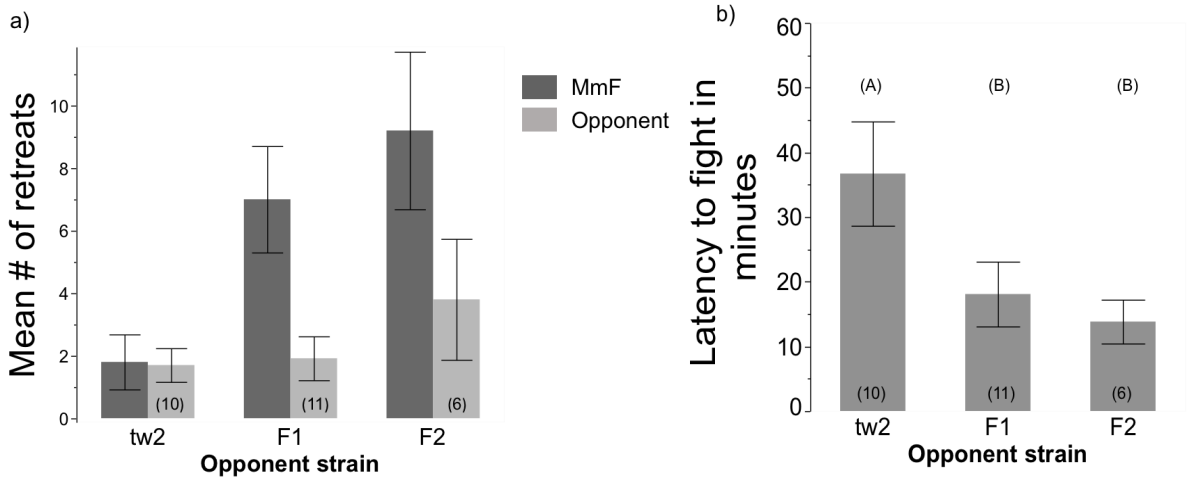

Figure 4. Male-male interactions in arena trials: a) Mean number of retreats by MmF male b) Mean time to first fight in minutes. Significant differences between groups indicated by different letters (Tukey's HSD). Sample sizes are indicated in parentheses.

We also see that hybrid F1 and F2 backcrossed opponent males pursued females more often than did t^{w2} males (1-way ANOVA, $F_{2,24}$=9.742, p=0.001 Figure 5a). But, pursuit of females by the Farallon males did not show differences across trials with different opponent male types. Consistent with higher rates of pursuit, opponent F1 and F2 backcrossed males also showed higher rates of copulation than t^{w2} males (1-way ANOVA , $F_{2,24}$=4.267, p=0.026, t^{w2}< (F1=F2), Tukey's HSD; Figure 5b).While Farallon males showed few copulations and no differences were observed across different opponent types (1-way ANOVA, $F_{2,24}$=0.284, p=0.744).

While Farallon males were competitive with t^{w2} males, fully wild-derived Farallon males were less competitive than hybrid males on several measures including retreat behavior, latency until first fight, and pursuits and copulations with females. If these males start with a disadvantage then perhaps this is why they are less competitive than hybrid males and sire fewer pups. These initial behavioral interactions may lead to the development of a hierarchy in which dominance and territories are established and remain stable for the duration of the trial (Mackintosh, 1981; Latham & Mason, 2004). The ability of a hybrid mouse that carries the t^{w2} to adapt readily to novel competitive situations would likely be critical for these males to become established on islands. Studies also indicate that mating order strongly influences reproductive success with males who mate first siring the majority of offspring (Firman & Simmons, 2008; Sutter & Lindholm, 2015). Consistent with these previous findings and the paternity findings in this study, the majority of copulations observed following introduction into arenas were by F1 and F2 hybrid males.

The inability of t^{w2} laboratory males to compete with wild-derived Farallon males was not unexpected, but it is interesting as C57BL6 mice are considered more defensive and

aggressive in response to perceived threats compared to other laboratory strains (Blanchard et al., 2009). Laboratory mice also tend to be more aggressive than wild populations and more likely to defend territories (Latham & Mason, 2004). The Farallon mice may also be less aggressive in nature as rodents on islands can exhibit 'island syndrome' (Adler & Levins, 1994). The island syndrome has been observed in mice and associated with lower levels of aggression, increased survival rates, and increased reproductive output (Adler & Levins, 1994; Gray & Hurst, 1998; Cuthbert et al., 2016). Differences in aggression may be related to whether mice are living commensally or not, and some previous studies indicated that increased density might also favor more aggressive individuals (Berry et al., 1991; Gray & Hurst, 1998). Although small in number, other seminal studies have strongly suggested that island house mice may not be as competitive as their mainland/commensal counterparts (Mackintosh, 1981; Berry et al., 1991; Gray & Hurst, 1998).

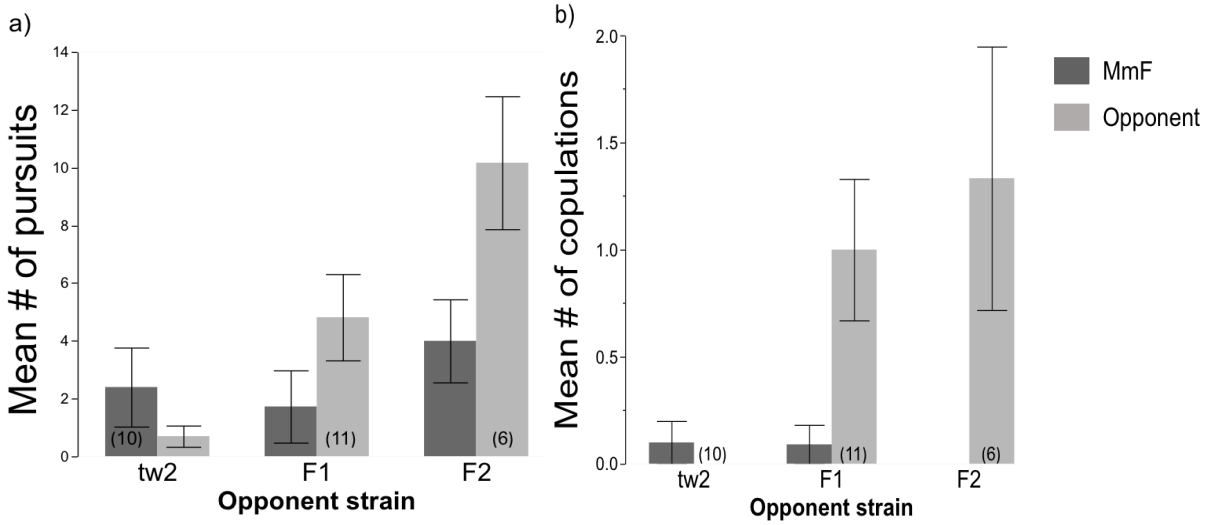

Figure 5. Male-female interactions in arena trials: a) Mean number of pursuits of females by males b) Mean number of copulation events. Sample sizes are indicated in parentheses.

Reproductive System Differences

The surprising result that hybrid F1 and F2 males were stronger competitors than Farallon males immediately following introduction in our arena trials led us to examine whether reproductive differences could account for these findings. Testes weights are correlated with dominance and mating success, as mice with greater testes mass are more likely to initiate matings with females and to attack rival males (McKinney & Desjardin, 1973). Since t^{w2} carrying males are larger than Farallon males, we used testes to body weight ratio as a conservative comparison measure of testes size. We also nested by age to see if this was a confounding variable. We found that F1 wild-lab mice had significantly larger relative testes size compared to the Farallon mice, while F2 wild-lab males had the second largest testes weights, with no effect of age (1-way nested ANOVA strain $F_{23,42}$=2.542, p=0.004, age interaction $F_{20,20}$=1.651 p=0.085, Tukey's HSD, $p<0.05$; Figure 7).We also weighed seminal vesicles as their weight and size have been linked to testosterone concentrations (Bartke, 1974: Le Roy et al., 2001). We found a significant effect for strain, but also for age indicating older males had larger relative seminal vesicle weights and that t^{w2} lab males had higher weights than Farallon males (1-way nested ANOVA strain $F_{23,42}$=3.520, $p<0.0002$, age interaction $F_{25,25}$=2.987 p=0.001, t^{w2} > MmF, Tukey's HSD; Figure 7). We also found that none of the mice were compromised in testes and/or seminal vesicle weight as they all are within the normal range for mice but there may be further differences in reproductive physiology not explored in this study.

The reproductive competitiveness of *t*-haplotype bearing males is important from both a basic standpoint and an applied context if used in a genetic pest management approach. For example, other studies examining other types of *t*-haplotypes found carrying the haplotype was detrimental to male success (Carroll et al., 2004; Lindholm et al., 2013). We decided to conduct

morphological analyses and testicular spermatid head counts to see if there were differences in the type and number of sperm for Farallon versus opponent males. There were no visible morphological abnormalities seen with any of the groups examined. For spermatid head counts, Farallon males had significantly lower spermatid head counts than t^{w2} males while the F1 and F2 hybrid males fell between the two (1-way ANOVA, $F_{3,104}=17.924$, $p< 0.045$: Tukey's HSD, Figure 8). These results indicate that the reproductive advantages of F1 and F2 males do not appear to be due to differences in sperm numbers. Likewise, the reproductive disadvantage of the t^{w2} lab males does not appear due to differences in sperm counts either as they had statistically higher spermatid head counts compared to Farallon males, but lower success.

Several previous studies provide evidence of female choice against *t*-haplotype bearing males (Lenington et al., 1994; Carroll et al., 2004; Manser et al., 2015) as well as a lack of *t*-bearing sperm competitiveness (Sutter & Lindholm, 2015). The Sutter and Lindholm study however, used a *t*-haplotype that is homozygous lethal and ours was the first to study sperm competition with a non-lethal *t*. Our pilot study examining sperm competition did not find a disadvantage to carrying the t^{w2}. A binomial logistic regression found that mating first significantly increases fertilization success, but carrying the t^{w2} did not affect mating success (Mating order Chi-square=6.91, df=1, p= 0.009, carrying t^{w2} Chi-square=0.32,df=1, p=0.570, n=12, Table 3). While the sample size was relatively small in this study, the lack of any apparent difference suggests the t^{w2} variant of the *t*-haplotype used here may not confer a similar disadvantage in sperm competition to the variant studied by Sutter and Lindholm. As noted above, the t^{w2} variant is homozygous sterile, but not lethal (Dunn & Levene, 1961; Lyon, 2003). It would be interesting to know if t^{w2} bearing sperm are less functionally compromised than other *t*-haplotype variants.

Conclusion

While our experiments intended to test if it was possible to create an engineered meiotic drive mouse that was competitive and could succeed on an island, the results provided information on basic questions about wild and lab mouse behavior in larger arena settings. We confirmed that strong transmission ratio distortion of the t^{w2} variant of the *t*-haplotype also occurs in a wild-derived background. This would be critical for using the *t*-haplotype with the masculinizing gene *Sry* inserted to sex bias offspring and reduce invasive mouse populations on islands. We also found that hybrid F1 and F2 wild-lab mice can successfully outcompete wild-derived mice, at least in our arena settings, with more than 80% of offspring sired by these hybrid males. We found that the greater success of these hybrids could not be accounted for by differences in body size, relative testes and seminal vesicle weights, spermatid head counts, or sperm competition. Importantly, the greater competitive success of F1 and F2 males did correlate with several behavioral differences evident following introduction to the arenas. These include reduced latency to fight and increased female pursuits and copulations.

Reproductive fitness would be critical for an engineered mouse. An engineered mouse with the *t*-*Sry* could carry fitness costs due to the construction and insertion of the *Sry* gene into the *t*-haplotype (Backus & Gross, 2016). Modeling efforts to date have focused their attention on the potential fitness costs for an engineered organism (Champer et al., 2017; Prowse et al., 2017; Dhole et al., 2018), yet our results suggest modeling should also address the possible benefits of increased ability in reproductive competition. A mouse that is reproductively competitive may help compensate for any potential fitness costs associated with an engineered *t*-*Sry* construct. Importantly in this context, a study by Berry and colleagues (1991) found that male mice introduced from a commensal population were able to introgress into a wild island population.

House mice were removed from the Orkney Isle of Eday and released onto the Isle of May, which had an existing invasive mouse population. Using a Y-linked genetic marker unique to the Eday population, these researchers found that the marker spread through and replaced the Y-linked allele from the Isle of May population (Berry et al., 1991: Jones et al., 1995). A follow up laboratory study by Gray and Hurst (1998), found that these Eday males were considerably more aggressive and behaviorally dominant to the Isle of May males. Carefully assessing the reproductive competitiveness of any engineered strain will likely be critical to the success of a genetic approach to invasive rodents on islands.

A key limitation of the current study stems from the size of the arenas used and the consequently small number of individuals in a trial. An important next step will be to scale up trials in arena size and environmental complexity. Increased enclosure size will allow for increased population sizes, the formation and defending of larger territories, development of naturalistic social hierarchies, as well as testing across multiple generations. While rodents are difficult to observe in large and complex environments, new approaches using Radio Frequency Identification (RFID) systems allow detailed and continuous monitoring over longer time periods (Weissbrod et al., 2013; Auclair et al., 2014; König et al., 2015; Lopes et al., 2016). RFID tracking would allow further detailed collection of behavior beyond the first hour following introduction examined here.

New approaches are needed for eradicating invasive rodents on islands (Campbell et al., 2015) and a strongly interdisciplinary approach is necessary (GBIRd, 2018). As scientists continue to develop a *t-Sry* mouse for this purpose, it is also critical to continue behavioral experiments extending the results presented here and related efforts in the area of modeling (Backus and Gross, 2016; Prowse et al., 2017; Sudweeks et al., *in review*). Indeed, Moro et al

(2018) identified the translocation of conspecifics into existing populations as a key knowledge gap for application of gene drive research addressing invasive house mice. Lastly, we agree with Burt (2003) and others that while any application of gene drive technology is still in the development stages, it is critical to address social and ethical questions as well.

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Supplementary Methods

a) Testes and Seminal Vesicle Weights

Male mice were euthanized and a full body weight in grams was measured. Testes were removed as were the seminal vesicles and weighed to the nearest 0.01mg. To prevent the loss of seminal fluid each seminal vesicle was grasped with forceps before carefully excised of adhering tissue. Testes and Seminal vesicle weights were calculated relative to body weight and nested by age with 1-way ANOVA's, followed by Tukey's HSD multiple comparisons test to characterize differences between groups.

b) Testicular spermatid head counts

Spermatid head counts were derived using frozen testis with a method modified from Seung et al. (2012). Briefly, right and left testis were removed and frozen at −70°C until tested. Each testis was then thawed, and the tunica albuginea was removed. Following tunica removal, the tissue was homogenized with a dimethyl sulfoxide (DMSO) and saline solution (10% DMSO, 0.9% NaCl), centrifuged, and then resuspended in the same solution. The suspension was then stained with a 0.1% trypan blue solution (Fisher Scientific). Sample aliquots of 10µl were then placed on a hemocytometer and counted under 100x magnification. To assess consistency of counts, paired right and left testis were compared for 11 individuals and no significant differences were detected. A 1-way ANOVA followed by Tukey's HSD multiple comparisons was used to characterize differences between groups.

c) Sperm competition

The protocol used to assess sperm competition is originally from Firman and Simmon (2008) and was modified by Suter & Lindholm (2015). This protocol was then further modified

for this study. To address potential *t*-haplotype effects on success in sperm competition, we created F1 wild-lab sibling pairs of brothers in which half of these males carried the t^{w2} by pairing t^{w2} lab females with a Farallon male. We then paired nulliparous C57BL/6J females with the F1 wild-lab males and varied the order of those carrying the *t*-haplotype with 50% being the first male and 50% being the second male. Prior to mating, females were checked for estrus based on visual features and vaginal cytology. Females in proestrus or estrus were paired with a non-related F1 male in the female's home cage at approximately 5:00pm. Females were checked for the presence of a copulatory plug every 1-1.5 hours. After a copulatory plug was detected, the plug was removed, and the second male was then placed into the female's cage. The females were again checked every sixty minutes for a mate plug or until the following morning. The females were maintained in separate cage to produce pups. Tissue samples were taken from pups on the day of parturition to assess t^{w2} transmission as described above. Sperm competition was assessed statistically via binomial logistic regression for the number of pups born with and without the *t*-haplotype. Of note, we attempted to use Farallon females for sperm competition studies, but were unable to find a sperm plug following mating and so C57BL/6J females were used instead.

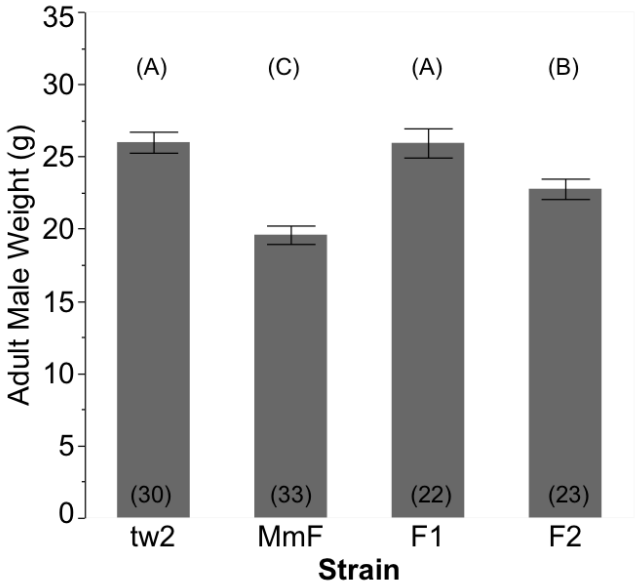

Figure 6. Male adult weight by strain. Significant differences in weights indicated by different letters (Tukey's HSD). Sample sizes are indicated in parentheses.

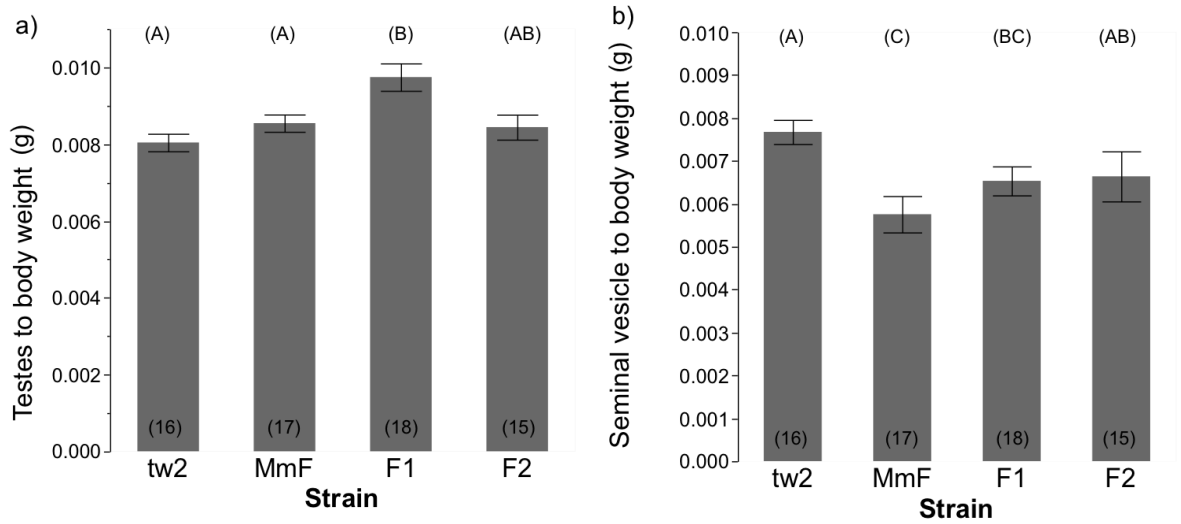

Figure 7. Mean testes and seminal vesicle to body weight ratios: **a)** Mean testes to body weight ratios by strain. **b)** Mean seminal vesicle to body weight ratios by strain Significant differences in mean weights indicated by different letters (Tukey's HSD). Sample sizes are indicated in parentheses.

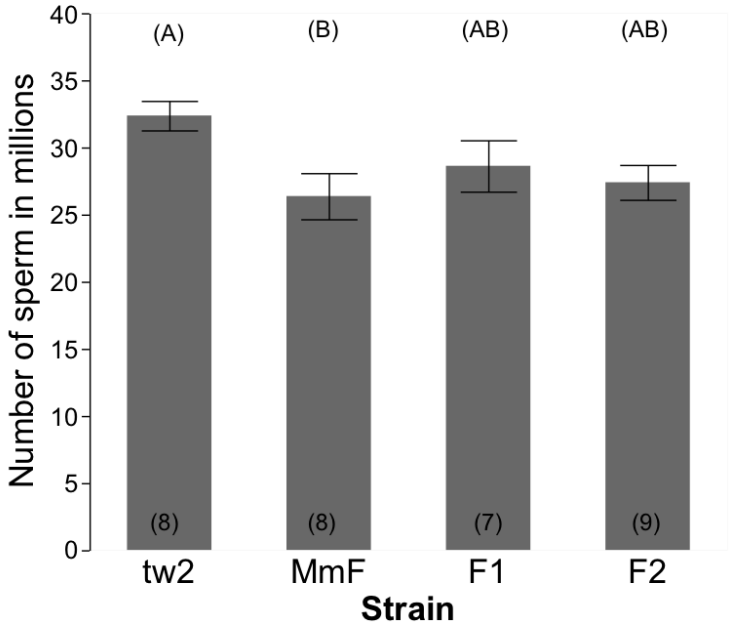

Figure 8. Mean spermatid head count in millions compared across strains. Significant differences in weights indicated by different letters (Tukey's HSD). Sample sizes are indicated in parentheses.

Table 3. Sperm competition outcomes for F1 males with or without the t^{w2}, the mating order, number of pups produced, and the percentage bearing the t^{w2}.

Mating Order	**Number of pups**	**Percent with t^{w2}**
non t first	4/4	100% t
non t first	8/8	100% t
non t first	0/8	0%
non t first	0/6	0%
non t first	0/7	0%
non t first	0/5	0%
t first	8/8	100% t
t first	0/7	0%
t first	0/4	0%
t first	5/7	71%
t first	2/2	100%
t first	8/9	89%

Supplementary Reference

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Chapter 4

Scenario Analysis on the use of rodenticides and sex-biasing gene drives for the removal of invasive house mice on islands

Abstract

Since the 1960s conservation efforts have focused on recovering island biodiversity by eradicating invasive rodents. These eradication campaigns have led to considerable conservation gains, particularly for nesting seabirds. However, eradications are complex and lengthy endeavors and are even more challenging when humans are co-inhabitants of the targeted island. Furthermore, the method of eradication matters and recent proposals to consider genetic technologies for rodent eradication require specific scrutiny. One such technology is the potential use of a gene drive for biasing offspring sex ratios in invasive house mice, *Mus musculus*, that would spread and prevent the production of one sex, allowing die off from lack of reproduction and natural attrition. We can gain insight into the potential for adoption of this technology from examining stakeholder engagement. This paper uses scenario analysis to address the eradication of rodents on inhabited and uninhabited islands, by specifically comparing the traditional approach of using rodenticides with sex-biasing gene drives. Concurrently the International Union for Conservation of Nature is assessing the risks and value of gene drives in general for conservation. Hence, we make the case that the ethical challenges with the use of gene drive sex-biasing techniques and the effectiveness of this tool will rely as much on its public acceptance and its democratic use as the actual science used to construct the technology.

Introduction

Island biodiversity threatened by invasive rodents

Islands are only 5% of our terrestrial land mass, but 37% of all critically endangered species are endemic to islands (Tershy et al., 2015). Furthermore, invasive species have a disproportionate impact on island ecosystems (Bellard et al., 2016). In particular, 80% of the world's islands harbor invasive rodents; the most common are house mice (*Mus musculus*) and three species of rats (*Rattus rattus, R. norvegicus, R. exulans*) (Caut et al., 2008). These invasive rodents are nearly ubiquitous and often negatively impact native fauna and flora (Mulder et al., 2008; Drake & Hunt, 2009). Islands are the nesting and breeding grounds for seabirds, and rodents can have devastating impacts, consuming eggs and chicks, and gnawing on adults (Fukami et al., 2006; Caut et al., 2008). Scientists have demonstrated that removing invasive rodents increases seabird reproductive success (Capizzi et al., 2014; Jones et al., 2016). In addition, it was recently discovered that coral reefs can benefit from the eradication of invasive rodents as seabirds enhance coral reef productivity (Graham et al., 2018). Another indirect effect occurred on Palmyra Atoll when invasive rats were eradicated the invasive Asian tiger mosquito (*Aedes albopictus*), a known human disease vector, was secondarily extirpated (Lafferty et al., 2018). On populated islands, rodents are responsible for large field and storage losses to economically important crops such as maize, soybean, and rice (Brown & Singleton, 2000). Rodents can also transmit diseases such as Leptospirosis directly to humans and can indirectly transmit other diseases (Vanasco et al., 2003).

Rodenticide Eradications

The primary means of removing invasive rodents from islands is rodenticides (Campbell et al., 2015). Rodenticides are anticoagulants that cause death when ingested and are placed into bait which is either spread aerially or maintained at bait stations (Capizzi et al., 2014). The first successful rodent eradication campaign occurred in New Zealand on Maria Island in 1964 (Russell & Broome, 2016). The use of aerial baiting has increased the size and scope of these efforts to South Georgia island (30,000ha), in the southern Atlantic ocean , which represents the largest campaign to date and the island is now rodent free (Simberloff et al., 2018). For aerial campaigns, native species of concern must often be captured and contained for months -- or even years -- to prevent accidental death by ingestion (Howald et al., 2010). Modern rodenticides are second generation anticoagulants which are slower acting, prevent bait shyness, and allow the rodents to consume multiple rounds (Howald et al., 2007; Capizzi et al., 2014). Currently over 650 rodent eradication campaigns have been launched worldwide with the majority being successful at eradicating rodents with a less than 20% failure rate for those attempted overall (Holmes et al., 2015a).

Rodenticides have drawbacks: they are not species-specific, non-target species may accidentally consume the rodenticide, and resistance can occur (Parkes et al., 2011; Campbell et al., 2015; Rueda et al., 2016). On inhabited islands, eradication is particularly challenging because of the potential for accidental human or livestock consumption (Oppel et al., 2011; Glen et al., 2013; Campbell et al., 2015) and complete eradication is not always feasible requiring repeated efforts (Howald et al., 2007; Angel et al., 2009).

From inception to implementation a campaign can take 5-10 years (Howald et al., 2010). Primarily conservation needs and priorities determine the decision on where to attempt a

campaign (Brooke et al., 2007). Island eradication campaigns have been carried out through the coordination with land managers and non-governmental organizations such as Island Conservation (Island Conservation, 2018). These campaigns are labor and cost intensive and highly dependent on several factors: island size, remoteness, native species present, whether it is inhabited with people, and their support or lack thereof for the campaign (Holmes et al., 2015b). Because of these concerns and limitations, many conservation practitioners have stated they are quickly running out of islands that can be restored using current methods (Goldson et al., 2015). This situation, along with ethical concerns about using rodenticides, has led to a push for the investigation of newer technologies, such as sex-biasing gene drives (Campbell et al., 2015; Goldson et al., 2015; Sutherland et al., 2018).

Sex-Biasing Gene Drive Strategies

Gene drives are a potential rodent eradication methodology that would avoid use of rodenticides. Gene drive methods draw on sterile insect technique methods developed in the 1950s (Knipling, 1955) and refined by Oxitec to develop genetically-engineered mosquitoes (Capurro et al., 2016). Gene drives are genetic constructs that are selfishly inherited by the majority of offspring and can be used to spread a particular trait into a population. Unlike the traditional Mendelian ratio of a fifty percent chance for inheritance, gene drives can potentially transmit to offspring at near 100% ratios and are modeled as a potential pest control tool (Burt, 2003; Sinkins & Gould, 2006; Esvelt et al., 2014). Some gene drives exist in nature while others are synthetic. Synthetic gene drives have been demonstrated in mosquitoes, flies, worms, and yeast (DiCarlo et al., 2015; Esvelt et al., 2014; Gantz & Bier, 2015; Harris et al., 2012). Modeling suggests that these gene drives could be engineered to spread to all members of a

particular population from the release of a few individuals (Harvey et al., 2017). Sex biasing gene drives are being researched and considered for rodent eradication whereby the sex ratio of the population would be biased, leading to local extirpation due to a lack of suitable mates (Leitschuh et al., 2017; Piaggio et al., 2017a) (Figure 1).

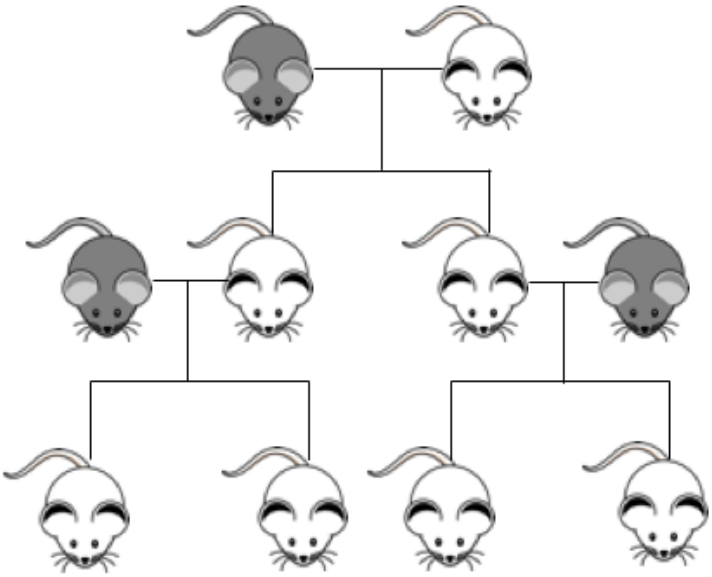

Figure 1: Depiction of how a sex biased gene could spread through a population. The grey color depicts the wild-type and the white is the gene drive.

While gene drives are considered species-specific and non-lethal (Harvey et al., 2017), they present other environmental concerns. Gene drives are designed to be self-sustaining and could spread beyond the targeted geographic region if control measures are not in place (Moro et al., 2018). In addition, there is a small risk that gene drive rodents could mate with heterospecifics and spread via hybridization to populations of rodents that are native and beneficial to the ecosystem (Harvey et al., 2017). In 2016 the National Academy of Sciences, Engineering, and Medicine called for caution in studying gene drives in the laboratory and urged

phased testing throughout the entire project (National Academies of Sciences, Engineering and Medicine, 2016). Similar reports have been developed in Australia and New Zealand (Australian Academy of Science, 2017; Royal Society Te Apārangi Gene Editing Panel, 2017). Scientists in the USA, Australia, and New Zealand are examining the potential use of sex-biasing gene drives in house mice working within biosecure facilities (National Academies of Sciences, Engineering and Medicine, 2016; Australian Academy of Science, 2017; Royal Society Te Apārangi Gene Editing Panel, 2017). A future goal, however, is to release these altered mice on an uninhabited island as part of carefully monitored field trials, in a country with a robust regulatory environment, perhaps in one of the three above mentioned countries (Genetic Biocontrol of Invasive Rodents, 2017).

Public Perceptions of Rodents

People who live with rodents often express concerns about rodents' effects on food-stocks, clothes, and overall human health, and want the rodents removed (Reiter et al., 1999; Morzillo et al., 2011; Panti-May et al., 2017). However, there is sometimes opposition to removal, motivated by concerns towards the target animal, the humaneness and effectiveness of control methods, unknown environmental impacts, ability to target specific animals, and cost (Dubois et al., 2017; Fitzgerald, 2009; Reiter et al., 1999). Animal welfare and the number of organisms to be killed are points of contention (Dubois et al., 2017). Opponents report strong ethical concerns about killing for conservation and generally prefer no-kill methods (Courchamp et al., 2017). The results of a survey that measured New Zealander's perceptions of lethal methods for wildlife control indicated that 'poisoned baits for rodents' is an acceptable form of lethal control (Reiter et al., 1999). A US-based study that measured perceptions of lethal

methods found respondents to be most concerned about species specificity, pain level, and efficiency of method (Sanborn & Schmidt, 1995; Fitzgerald, 2009). A study of news media coverage of rodent eradications indicated that media coverage of eradications was supportive of eradications, although a majority of the islands addressed were uninhabited (Valdez et al., 2019).

Important here are the bases for opposition to eradication. Managers often attribute opposition to ignorance, but studies show that public education designed specifically to garner public support does not generally increase acceptance and can heighten conflict (Owens, 2000; Crowley et al., 2017). Therefore, public engagement campaigns should be at least as much about public deliberation as they are public education.

The range of cultural perspectives about rodents as invasive species is also often overlooked. Some Indigenous Australians do not view invasives as incompatible with native species, or the invasive may be viewed as a resource (Fitzgerald, 2009). The Maori of New Zealand view the Pacific Rat (*R. exulans* or Kiore as sacred) (Haami, 1994). Moreover, the majority of rodent eradications have occurred on wealthy islands and there is concern that eradication managers may dismiss or overlook groups of people with less privilege (Brown et al., 2017). Some people will never support an eradication campaign. Others argue it is the only way to save endangered species. Thus, stakeholder groups have shifting social dynamics that eradication managers need to address, acknowledge, and adapt to (Morrison et al., 2011; Crowley et al., 2017; Dubois et al., 2017; Novoa et al., 2018).

Governance of Gene Drive Rodents for Conservation

There are a number of regulatory institutions that could regulate gene drive rodents. In the United States, for example, eradications are regulated by the National Environmental Policy

Act (NEPA), which requires an Environmental Impact Statement (EIS) as a decision-making tool (Meghani & Kuzma, 2018). Within this framework, NEPA uses quantitative risk assessments to evaluate courses of action (including no action). Similarly, the Coordinated Framework for the Regulation of Biotechnology also relies on "verifiable scientific risk" as the basis for decisions made about the release of genetically engineered organisms (Office of Science and Technology Policy, 1986; Kuzma, 2016). Because both NEPA and the Coordinated Framework regulate shared environments, mechanisms for public input are included as explicit dimensions of the decision-making process. For example, NEPA would require an EIS and public comment period for sex-basing gene drives (Hayes et al., 2018), and genetically engineered organisms also require public comment periods (Kuzma, 2016). Similarly, Australia's Environmental Protection and Biodiversity Act (EPBC Act) also requires risk assessments when examining wildlife management strategies and encourages public input (EPBC, 2018; Hayes et al., 2014). New Zealand's Royal Society Te Aparangi and Landcare Research have been investigating the potential of gene drives with a panel that solicits public input (Royal Society Te Apārangi Gene Editing Panel, 2017; Dearden et al., 2017). From a global perspective, the UN's Convention on Biological Diversity is currently reviewing language to address the governance of gene drives (Callaway, 2018a). However, the United States never ratified the Convention, and while the US continues to send delegations to the relevant negotiations, the absence of a binding commitment will likely add to the complexity of regulating gene drives (Oye et al., 2014).

Because emerging gene editing technologies outpace the regulatory structures that govern them, scholars and stakeholders have drawn attention to the *governance* of these technologies (Kofler et al., 2018; Kuzma, 2016). Governance -- distinct from governments -- would broaden the scope of responsibility of oversight from mandatory government regulations to also include

voluntary standards or norms that address scientific ethics and respect for impacted communities (National Academies of Sciences, Engineering and Medicine, 2016). One important feature of more inclusive governance processes is the nature and role of public input into the decision-making ecosystem. Public comment periods that are built into regulatory systems (described above) are important starting points for soliciting public input, but may not account for the complexity associated with gene drive rodents. As such, more deliberative processes are needed.

One governance approach that can attend to complex perspectives, and is consistent with calls in the governance of emerging gene editing or biotechnologies, is deliberative community, stakeholder, and public engagement (National Academies of Sciences, Engineering and Medicine, 2016). Communities are generally defined by geographic proximity to project impact, while stakeholders have some professional or personal interest in the issues. Public audiences may not be characterized by the same geographic or professional connection, but they are comprised of individuals who have some interest or concerns. Additionally, we use the definition of engagement from the National Academies of Sciences, Engineering and Medicine gene drive report: "seeking and facilitating the sharing and exchange of knowledge, perspectives, and preferences between or among groups who often have differences in expertise, power, and values" (National Academies of Sciences, Engineering and Medicine, 2016 p. 132).

An important feature of more deliberative engagement is the potential to discuss technological innovation, their potential hazards and benefits, and the values that underpin varying positions (Kuzma et al., 2018). In these cases, more deliberative public engagement efforts, such as workshops or citizen/stakeholder advisory committees will likely be better suited for making decisions regarding invasive rodent management (Chess & Purcell, 1999). These public engagement efforts will likely require more time and resources, but there are a number of

reasons to invest in these types of engagement efforts. Citizens should have a right to know, and a right to participate in decisions that impact their lives (Cox, 2012). Additionally, deliberative public engagement offers a potentially better process for incorporating input because participants have a space to communicate their preferences and better understand the preferences of other participants (Parkins & Mitchell, 2005). Finally, deliberative public participation can lead to better outcomes, or better eradication plans in this case, because multiple stakeholder perspectives are discussed, knowledge can be shared between parties and consensus or near-consensus decisions can be developed (Parkins & Mitchell, 2005; Walker, 2007).

Scenario Analysis

In this section we analyze four scenarios that illustrate the complexity of eradications. A scenario analysis can help organize insights into a framework that integrates qualitative and quantitative information, and gauges risks (Swart et al., 2004). Results of scenario analyses can be communicated to broad audiences and can provide guidance for planning (Swart et al., 2004). With this in mind, we present the following scenarios to demonstrate the differences between applying rodenticides and gene drives on uninhabited and inhabited islands. We draw attention to the ecological, financial and social contexts that are important to consider when developing rodent eradication proposals on similar islands.

Eradication with rodenticides on an uninhabited island

The Antipodes are a known nesting site for 21 species of seabirds with house mice being the only invasive mammal (Elliott et al., 2015). Nicknamed the “Million Dollar Mouse Project”, the 2016 invasive house mouse (*M. musculus*) eradication on the Subantarctic Antipodes

(2045ha) marks a milestone for mouse eradication in terms of island size (Russell & Broome, 2016: Wickes, 2016: Million Dollar Mouse Project, 2018). The campaign used 65,500kg of rodenticide-laced bait to remove an estimated 200,000 mice from the island (Wickes, 2016). The project's planning phase began in 2012 and was deemed a success in 2018 when mice were no longer found, after the typical two year monitoring period (Million Dollar Mouse Project, 2018). The cost of the aerial eradication was around 2.6 million USD, with New Zealand Government, Morgan Foundation, World Wildlife Foundation, Island Conservation and New Zealanders contributing funds (Wickes, 2016). Now that the mice have been eradicated, biosecurity measures need to be in place to prevent reinvasion, requiring more time and expenditure (Russell & Broome, 2016).

Eradication with rodenticides on an inhabited island

Australia's Lord Howe Island (2,100ha) may become the largest human-inhabited island to eradicate rodents. Popular among tourists, the island support an abundance of unique fauna including an endemic giant stick insect, *Dryococelus australis* (Priddel et al., 2013). Lord Howe's year-round population is approximately 350, but doubles with tourists (capped by the island governing board at 400) (Oppel et al., 2010; Reis & Hayward, 2013; Cavanagh, 2018). Greater than 75% of Lord Howe is a permanent park preserve aimed at protecting and preserving land and is also listed as a world heritage site by UNESCO (Reis & Hayward, 2013). Unfortunately, rodents negatively impact over 70 plant and animal species on the island and have already caused the extinction of several species of birds, insects, and plants on the island (Wilkinson & Priddel, 2011). In accordance with existing Australian laws and governance, the eradication will need to account for the safety of the 350 year-round residents (along with their

pets and livestock) and may consider a limited ban on tourism (Oppel et al., 2010; Wilkinson & Priddel, 2011). Similar to previous eradications using rodenticide, the Lord Howe Island eradication would require the temporary housing of two bird species of special concern, the Lord Howe Woodhen (*Gallirallus sylvestris*) and Lord Howe Currawong (*Strepera graculina crissalis)* (Lord Howe Island Rodent Eradication Project, 2017). Currently, the project is estimated at 7 million USD (Gillespie & Bennett, 2017) but delaying the eradication can drive up costs (Russell et al., 2018). Cost benefit analyses that compare current methods of rodent management to the eradication campaign have been made public to show that the eradication could financially benefit the island (Gillespie & Bennett, 2017). The Lord Howe Island Board has allowed for an open public comment period in accordance with Australia's EPBC Act (Lord Howe Island Rodent Eradication Project, 2017). Lord Howe Island Eradication managers have also conducted social impact assessments to address key issues and are hoping the eradication will proceed. The 2015 referendum was voted marginally in favor but plans for 2018 have been delayed to 2019 (Russell, 2018). The eradication of mice and rats has been delayed several times before due to the community's opposition to the campaign (Wilkinson & Priddel, 2011; Tolj, 2016: Cavanagh, 2018). Oppositional voices have expressed concern over potential risks to endemic species, human health, livestock and potential impacts to tourism (Wilkinson & Priddel, 2011; Gillespie & Bennett, 2017). Lord Howe would demonstrate that eradications could be performed on inhabited islands, but also shows that eradications are more complicated when they involve people who reside on the island.

Eradication with a gene drive on an uninhabited island:

Sex-biasing gene drives in mice are still in development (Callaway, 2018b; Cohen, 2018) but will likely require unique planning and assessment efforts before being used in the field. First, the viability of gene drive mice needs to be addressed. Gene drive mice that succeed in laboratory environments may not survive and reproduce in the wild if the fitness cost of a drive mechanism is too high or if wild populations develop resistance to the gene drive (Manser et al., 2015; Champer et al., 2017; Prowse et al., 2017; Sudweeks et al., *in review*). Another major concern for rodent gene drives is their potential ability to penetrate and spread to unintended populations (Esvelt et al., 2014; Leitschuh et al., 2017). Scientists need to better understand and limit off-target effects, to ensure that the drive will not spread to other species, or spread outside the intended uninhabited island location (Esvelt et al., 2014; National Academies of Sciences, Engineering and Medicine, 2016). Methods to contain gene drives both temporally, spatially, and molecularly are being designed and considered (Leitschuh et al., 2017; Sudweeks et al., *in review*).

To prevent the unwanted escape of gene drive organisms, or even the intentional spread, control measures will need to be established and enforced. Methods and lessons on control can be gained by studying islands post rodenticide eradication and the majority of this work has been conducted in Australia and New Zealand (Greenslade et al., 2013; Russell & Broome, 2016). Researchers have suggested that a remote offshore island might be the best location to trial a gene drive sex biasing mouse as the uninhabited island provides physical containment as recommended by Champer et al (2016). Additionally, if the gene drive were to fail, existing regulations should permit following up with rodenticides as a failsafe.

There are ecological concerns related to rodent gene drives as well. If not timed properly a release could temporarily add more invasive mice to the population, which might have cascading ecological consequences, including increased competition for food resources, and increases in predation pressures (Esvelt et al., 2014; Backus & Gross, 2016; Esvelt & Gemmell, 2017).

The cost of a gene drive mouse is as of yet unknown, but development and upfront costs would be in the millions (Backus & Gross, 2016; Leitschuh et al., 2017). It is also important to note that the same monitoring and biosecurity costs to prevent reinvasion would still be present, but that there would not be the additional cost of housing threatened species off the island since a gene drive mechanism would be species-specific.

Deliberative public engagement will be important for an uninhabited island because the application of gene drive mice will be novel, complex, and it is difficult to predict how people will react to this method. In a nationwide survey of New Zealanders, 32% were comfortable with gene drives being deployed to control invasive species and 18% believed that gene drives should not be used leaving 50% undecided (MacDonald, 2017). In a survey of US residents, 37.6% of respondents disagreed stating it is not morally acceptable to edit genes to control invasive species, 30.1% neither agreed or disagreed, and 32.3% agreed it is morally acceptable, though not all participants said they understood how a gene drive functions (Brossard et al., 2018). In short, public opinion about gene drive technologies is complex and divided. Opposition to sex-biasing gene drives may be based on perceptions of what is natural, and how much humans should interfere with nature (Redford et al., 2014; Piaggio et al., 2017; Brossard et al., 2018). Additionally, the development of methods to contain gene drives temporally, spatially, and molecularly may actually erode public trust because people may become concerned that if gene

drives potentially need controls then the gene drive organisms should not be released. There has also been some concern that uninhabited islands might be viewed as more 'pristine' and that we should not interfere with areas 'untouched' by humans.

Eradication with a gene drive on an inhabited island:

Currently, there are no known plans to release a gene drive mouse on any island and a logical first step would be a remote uninhabited island (as discussed above). However, we discuss the inhabited island scenario because the planning for this type of eradication will require considerable foresight because an inhabited island will likely be more ecologically and socially complex. The ecology of urban rodents differs from rodents on uninhabited islands. Non-commensal rodents on islands have been documented eating a higher number of invertebrates as opposed to grasses and on seasonal islands they can dramatically increase in number during periods of food abundance (Angel et al., 2009; Backus & Gross, 2016). Commensal rodents also may behave and move differently than those living on islands non-commensally (Gray & Hurst, 1998). The regulatory structure for gene drive mice on an inhabited island is not likely to fundamentally differ from regulations on an uninhabited island. However, assessments for impacts to domestic animals and livestock will likely be necessary to build relationships with residents, even if risks seem minimal (Ogden & Gilbert, 2011).

The justifications for eradicating invasive rodents from an island will affect public perceptions, and this is likely to be particularly true for a genetic approach. However, a no-kill method of rodent eradication (such as gene drives) may be preferred over rodenticides (Campbell et al., 2015; Leitschuh et al., 2017). In addition to biodiversity losses, rodent populations can boom in agricultural areas. In Australian agricultural environments, invasive mice reach

"plague" densities, at over 2,000 per hectare, causing severe financial and emotional hardships for farmers (Singleton et al., 2001). Concerns for rodents are often even higher in cities either because the desire to remove rodents is stronger or there is more educational information on costs of rodents to human health and food security. (Morzillo, 2011; Garba et al., 2014; Panti-May et al., 2017). People may not be as willing to deploy sex biasing gene drives for conservation purposes as they are for rodents impacting human health as support for genetically modified organisms is generally higher in the context of human health (Widmar et al., 2017; Funk & Hefferon, 2018).

Developing trust with island residents becomes a critical management goal in this scenario, especially if a gene-drive system is deployed over several years (Backus & Gross, 2016). This is because residents will become active eradication partners and their cooperation will be necessary for success. At a minimum, they can help eradication efforts by excluding rodents from food sources and minimizing available nesting sites in personal homes and other buildings. Residents will be more likely to cooperate if they trust the management agency and believe the eradication can be successful (Stern & Coleman, 2015). Deliberative public participation methods can help foster relationships and lead to eradication plans that are acceptable to stakeholders and communities (Parkins & Mitchell, 2005).

Conclusion:

Rodent eradications are complex and no technique, including sex-biasing gene drives, offers a "silver bullet". Any method may fail to eradicate pest species and people will be left with the conservation and economic burden of invasive species. Yet, inaction also leaves these same burdens. The above case studies introduce the complexity of eradicating invasive rodents

on islands. They also highlight the human side of conservation. We feel strongly that for any rodent eradication project, eradication campaigners must carry out a genuine, thorough public engagement process.

Despite much discussion on gene-drive sex biasing technology for rodents on islands, presently only one group is exploring the technique in house mice, the Genetic Biocontrol of Invasive Rodents Program (GBIRd, http://www.geneticbiocontrol.org/). The program involves seven global institutions exploring all aspects of the technique: science, safety, stakeholder engagement, regulation (Genetic Biocontrol of Invasive Rodents, 2017). Drawing on responsible research and innovation (Stilgoe et al., 2013), this group is working together to build a transparent innovative process that engages stakeholders early and often. While explorations of rodent gene drives are starting with house mice, it is also foreseeable that in the future this organization or others expand into gene drives targeting rats, which are more damaging to island flora and fauna (Towns et al., 2006).

If gene drive mice are determined to be a viable approach, eradication planners will need to navigate uncertain and potentially evolving biotechnology regulations. In the United States, the Coordinated Framework for the Regulation of Biotechnology does not provide clear insight into how a gene drive mouse would be regulated (Kuzma, 2016), but regulation may fall to one of the following three regulatory agencies: Food and Drug Administration, Environmental Protection Agency, or the USDA's Animal and Plant Health Inspection Service (USDA-APHIS) (Office of Science and Technology Policy, 1986). The Australian Academy of Sciences has been exploring gene drives for conservation (Australian Academy of Science, 2017), while the Commonwealth Scientific and Industrial Research Organisation has been examining the regulatory aspects of sex-biasing gene drive mice (Commonwealth Scientific and Industrial

Research Organisation, 2018). For New Zealand, sex-biasing gene drive mice would fall under the Hazardous Substances and New Organisms Act and would be evaluated on a case-by-case basis by the Environmental Protection Authority (Royal Society Te Apārangi Gene Editing Panel, 2017). Given that sex-biasing gene drives could spread beyond national borders, these technologies may warrant further international regulations. On an international level, the Convention on Biological Diversity has continued to discuss and evaluate the safety and potential use of gene drives for conservation (Secretariat of the Convention on Biological Diversity, 2015). In conjunction, the International Union for Conservation of Nature has decided to formulate an IUCN Synthetic Biology and Biodiversity Conservation assessment with recommendations for the 2020 World Conservation Congress (International Union for Conservation of Nature: Development of an IUCN policy on Synthetic Biology, 2018).

Given the urgent conservation need and the limits of existing technologies, now is the time to discuss the potential of gene drive sex biasing mice as an alternative to rodenticides for island mouse eradication. As an emerging technology, it remains to be seen exactly how or if sex biasing gene drive mice will be effective as compared to traditional rodenticide-based approaches. Regardless of technology deployed, one of the greatest steps forward in terms of eradication has been the call for not only increased engagement, but in studying the social aspects of eradication for conservation. With over 460,000 islands globally, and invasive rodents present on the majority of these, society will need to decide whether to eradicate, and if eradicating, which method to use (UNEP-WCMC, 2018).

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Chapter 5

Conclusion

This dissertation focused on the matings of wild-derived and *t*-haplotype laboratory mice to begin understanding key differences in reproduction and mate competitiveness that could impact the effectiveness of a genetic pest management approach. Specifically, the goal was to address the applied question of whether it is possible to create an engineered meiotic drive mouse that could be competitive and succeed on an island. More generally, the results from these studies inform basic questions about wild-derived and laboratory mouse behavior and sexual selection in larger, more natural settings. Our lab was the first to pair any wild-derived mouse with laboratory t^{w2} bearing mice. My initial results led me to assess whether *t*-haplotype carrying males can reproduce successfully when in competition with wild-derived males. Below I address three primary aspects of this work. First, I will summarize the key findings of my dissertation research. Second, I will discuss the limitations of the current project and future directions. Third and finally, I will highlight some key knowledge gaps that will need to be examined before considering employing a sex-biasing gene drive on an island.

Key Findings

The first step in assessing reproductive competitiveness between wild-derived and laboratory mice was confirming that our t^{w2} laboratory mice would breed successfully with our wild-derived Farallon mice. Results of these matings in standard laboratory housing indicated that litter sizes are comparable and there are no significant differences in the sex ratios for pups born, nor in the time of gestation, but weaned pup and adult wild-lab hybrid mice are significantly heavier. These findings indicate that while adapted to different environments, the two strains of mice can still interbreed, and many reproductive parameters are quite comparable. Following initial matings of wild-derived, laboratory, and hybrid mice, I then confirmed that the

t^{w2} variant had strong transmission ratio distortion in a wild-derived background (96.5%) by conducting PCR genotyping of the Hba-4ps locus for over 600 mice. The mating of wild and lab strains in laboratory cages was an important first step in examining reproductive differences, but does not necessarily predict success in more naturalistic conditions. Therefore, I assessed whether *t*-haplotype carrying males can reproduce successfully when in competition with wild-derived males. We built larger arenas that allowed for more freedom of movement, formation of nesting areas, and the development of social hierarchies. Surprisingly, the results from these arenas using *t*-haplotype (t^{w2}) transmission rates indicated that our wild-lab hybrid males are stronger competitors than wild-derived males. Specifically, we found that hybrid F1 and F2 mice can successfully outcompete wild-derived mice, at least in our arena settings, with more than 80% of the offspring being sired by hybrid males. We also examined basic reproductive characteristics and found that the greater success of these hybrids could not be accounted for by differences in body size, relative testes and seminal vesicle weights, spermatid head counts, or sperm competitiveness. Yet, the greater competitive success of F1 and F2 males did correlate with several behavioral differences evident following introduction to the arenas. These included reduced latency to fight and increased female pursuits and copulations by these males. Taken together, my studies are proof-of-concept that a *t*-haplotype hybrid mouse may be reproductively comparable and behaviorally competitive with wild island mice. This is essentially a likely 'pre-requisite' for the employment of a genetic pest management approach.

Limitations and Future Directions

Any study has limitations and presents opportunities, often learned from hindsight or surprising results. Working with wild animals can be challenging and holding them in standard

laboratory housing was a new endeavor for all involved in the project. In addition, I was not able to collect as many wild mice from the Farallon Islands as I had originally hoped for, due to adverse weather conditions and the remoteness of the islands. As the Farallon mice are from a wild population (and therefore not allowed in a standard animal care facility due to disease concerns), I also ended up having to help build and create a facility to maintain our mouse colonies. This took time and planning, but proved fruitful in helping me learn how to build and properly house and care for wild-derived mice.

In terms of male competition, a key limitation of the current study stems from the size of the arenas used and thus the small number of individuals in a trial. Increased enclosure sizes would allow for large population sizes, the formation and defending of larger, more naturalistic territories, development of social hierarchies similar to those in the natural situation, as well as testing across multiple generations. Since completing my studies, the Godwin laboratory has built eighteen outdoor enclosures (4.9x6.1m) to hold local house mice. These enclosures could allow for an expansion of the male competition project, which would provide the opportunity to assess male competition and reproductive success under conditions more typical of island environments. Rodents are difficult to observe in large and complex environments, but new approaches using Radio Frequency Identification (RFID) systems allow detailed and continuous monitoring over longer time frames including during nocturnal active periods (Weissbrod et al., 2013; Auclair et al., 2014; König et al., 2015; Lopes et al., 2016). RFID tracking also could provide detailed collection of behavioral data beyond the first hour following introduction that I examined in Chapter 3. I piloted an Ultra High Frequency (UHF) RFID study to monitor mice continuously over extended periods, in the arenas used for male competition. Unfortunately, as the system was ultra-high frequency, the tags had to be worn on the outside, which was difficult

to maintain as the mice would chew off one another's collars after a period of time. My pilot study, however, indicated individual male mice move differently depending on strain type. Specifically, t^{w2} carrying laboratory mice move more frequently than Farallon wild-derived or hybrid mice (data not shown). Future work in the Godwin lab will include using a newer, non-high frequency RFID technology in our larger outdoor enclosures to monitor individual mouse movements over time.

One of the questions that I had hoped to address in my dissertation was "secondary invasion", wherein individuals that are introduced later are able to successfully introgress into an established population of the invasive species. This is an interesting basic question for invasive species and would be important in an applied sense for the use of a gene drive to reduce invasive populations. A particularly intriguing finding from phylogeographic genetic analyses is a suggested strong asymmetry in the ability of males and females to introgress into established populations on islands with males apparently being more successful in this introgression (Förster et al., 2009; Searle et al., 2009; Gabriel et al., 2010; Bonhomme & Searle, 2012; Jones et al., 2012; Jones & Searle, 2015). The process by which newly-arriving mice are able to successfully introgress into demes on islands is not clear. To address these questions, I attempted to compare Farallon wild-derived mice with a Gainesville mainland wild-derived population. The proposed experiments aimed to compare the ability of male and female Gainesville wild-derived mice to invade established Farallon demes. While I was able to obtain a colony of Gainesville wild-derived mice, they proved difficult to breed. PCR genotyping of the Hba-4ps locus revealed that half of the Gainesville males in our colony were homozygous for the t-haplotype and the remainder were heterozygous. Follow-up genetic screening found that this t-haplotype is not t^{w2}, but another non-lethal variant (data not shown). As this was a confounding factor and breeding

success was limited, I was unable to test for secondary invasion. An interesting focus for studies in outdoor larger enclosures would be testing the phenomenon of secondary invasion. Large enclosures would be well suited to this as they would allow for the establishment of a resident population within a natural environment with larger population sizes and social hierarchies over multiple generations.

Knowledge Gaps

Gene drive technologies have advanced greatly over the last five years and there is new interest in using CRISPR-based gene drives for rodent eradication on islands. The reproductive biology of wild-lab hybrid mice with a CRISPR based gene drive is unknown and may or may not prove different than the use of a *t-Sry* technique in terms of male competition. Researchers are also examining sex biasing towards an all-female population, but the relative effectiveness of biasing towards males or females remains to be tested.

The release of a gene drive mouse to extirpate invasive mice from islands is still in the concept phase. The translocation of conspecifics into existing populations was identified by Moro et al. (2017) as a key knowledge gap for application of gene drive research in invasive house mice. My dissertation focused on the use of the *t-Sry* meiotic drive and determined that hybrid males with the t^{w2} are likely to succeed in competition and mating. However, my studies only used one strain of island mice for creating hybrids and we are unsure of how mice on other islands behave, or how other gene drive types could potentially influence behavior. Further testing with secondary invaders, at larger scales, and using other strains of mice would be helpful in testing the ability of conspecifics to invade an already established population as successful invasion would be important for any gene drive approach.

Beyond the biological construction of a *t-Sry* mouse, it will be critical to understand as many other aspects of rodent biology as possible in this context. Scaling up trials and using simulated natural environments with gene drive mice can help to further characterize rodent behavior in sex-biased scenarios. Beyond enclosures, field trials will be essential and choosing the island on which to trial an eradication will be critical. Likewise, many aspects of island mouse biology will need to be addressed: population regulation, fitness and life-history characteristics, population genetics, and other ecological factors. Mathematical modeling critical to predicting factors such as the rate of release is ongoing, but understanding the biology of the rodent population on the island chosen can help parameterize these models. Further, there are aspects to containment, ecology, and regulatory frameworks that will need to be addressed before moving forward.

While attempting to understand how all the factors discussed above would affect the function of a sex-biasing gene drive on an island is important, perhaps the biggest knowledge gap is public acceptance towards this technology. Chapter four of my dissertation discussed scenario analysis and compared the traditional approach of using rodenticides to sex-biasing gene drives to draw attention to the ecological, financial, and social considerations that are important to consider when developing rodent eradication proposals. Scenario analysis shows that there could be differences in how rodenticides and gene drives are perceived, but what we do not know is whether gene drives will be preferred over rodenticides for conservation purposes. Members of GBIRd have reached out to stakeholders and are soliciting input into how to approach the topic through deliberative engagement. Social scientists have also pointed out that these are values-based decisions regarding conservation and what is natural (Redford et al., 2013; National Academies of Sciences, Engineering, and Medicine, 2016). Likewise, who gets to

decide on the use or deployment of these technologies is also important for their democratic use. While sex-biasing gene drive mice are still in a proof of concept phase, now is the time to be exploring these other aspects of a gene drive approach. As responsible innovators, both biological and social scientists agree that now is the time to begin exploring the acceptance and use of gene drive mice for conservation by the relevant publics (National Academies of Sciences, Engineering, and Medicine, 2016). The questions I addressed for my dissertation are only a subset of those for which knowledge is needed to move forward with this approach. In summary, the breadth of questions that still need to be addressed for gene drives highlights the value and perhaps even necessity for an interdisciplinary approach for moving forward responsibly with new genetic technologies.

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