Evaluating Biosecurity of Physical Containment at USDA Animal Facilities to Prepare for Genetically Modified Rodent Trials

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ABSTRACT: House mice and rats have been introduced to most countries and islands worldwide and represent serious threats to biodiversity, economic enterprise, and human health. Genome editing and gene drives are being explored as new genetic biocontrol methods to effectively suppress rodent pests. An essential step in the translation of this technology to field-ready tools is to administer breeding and behavioral trials with freely-interacting genetically modified (GM) rodents. Due to the potential impacts of unintended release of GM organisms, these trials will require biosecure animal facilities that are rigorously tested to ensure physical containment. This study was conducted to develop and evaluate the biosecurity of a physical containment facility for house mouse trials at USDA's National Wildlife Research Center. First, we conducted >20 trials with 75 wild-caught (non-GM) house mice to test their ability to escape from small containment units (0.35 m^2). During these trials that lasted >160 days, mouse behaviors and escape attempts were documented following exposure to attractants (high value foods and potential mates) as motivators for escape. Just two mice successfully breached containment during early trials, and both were from chewing small holes in plastic walls that allowed escape to the other side of the containment unit. In a second series of trials, we assessed containment efficacy in a large (24 m^2) arena intended to more closely replicate conditions of free-breeding wild mice. In these trials, mice were held in groups of six to 26 for up to 6 months. Across trials, only one mouse escaped the arena, an incident most likely attributable to human error during routine animal husbandry activities. This mouse was captured in the secondary containment (live-trap) within hours of breaching the primary containment. Overall, the containment strategy utilized here presents a robust design, with redundant containment mechanisms that should serve as a model for future behavioral trials using GM rodents. Additionally, our study highlights the need for rigorous staff training, careful attention to construction methods and materials, and adhering to biosecurity protocols to ensure the highest levels of containment that will be essential for testing efficacy of genetic biocontrols.

KEY WORDS: animal population management, behavior in containment, gene drive, genetic biocontrol, genetic engineering, *Mus musculus*, non-lethal pest control, preventing rodent escape

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INTRODUCTION

Invasive house mice (*Mus musculus*) and rats (*Rattus* spp.) have been introduced to many ecosystems worldwide and are among the most damaging rodents to agriculture, private property, and natural resources (Towns et al. 2006, Angel et al. 2009, Shiels et al. 2014, Witmer and Shiels 2018). Through mostly unintentional introductions by humans, these rodents occupy most continents and islands worldwide (Atkinson 1985, Angel et al. 2009, Towns 2009).

Containing mice so that they cannot access human structures and resources, and preventing house mice from entering areas where they may threaten desired resources, is challenging. House mice have amazing abilities to jump, squeeze under, and chew through a variety of materials (Pitt et al. 2011). Small barriers to prevent invasive rodents from accessing native and endangered species have been effective in Hawaii (Shiels and Drake 2015) and Puerto Rico (Shiels et al. 2022a). Despite high construction and maintenance costs, predator proof fences, for which animals as small as neonatal mice cannot pass through, have been successful in protecting natural resources from mouse damage in New Zealand and Hawaii (Scofield et al. 2011, Young et al. 2013). In laboratory settings, wild house mice can be successfully contained for months, housed both singly in cages and in free-ranging groups in large (24 m^2) pens (e.g., Shiels et al. 2022b). However, ensuring physical containment of genetically modified (GM) wild rodents is of extremely high importance, particularly when there is a potential for escaped GM animals reproducing with local wild (non-GM) populations.

Recent efforts to use genome editing (involving CRISPR/Cas9 technology) in mice to generate genetic biocontrols (Teem et al. 2020) underscores the importance of developing strict containment protocols for GM rodents used in laboratory trials. Use of gene drives (naturally-occurring or synthetic) is of interest in constructing a genetic biocontrol rodent because gene drives force certain genes to be passed on via reproduction. Because gene drive technology can be species-specific, and it is non-toxic, it is viewed as a promising new tool for invasive rodent eradications on islands (Dearden et al. 2017, Campbell et al. 2019, Godwin et al. 2019). Gene drives in rodents and other animals have not yet been trialed in the field but this is of interest to pest managers if environmental risk is low and the probability of effectiveness is high. In recent laboratory cage trials, scientists have used genome editing to spread a female fertility gene that could lead to local eradication of mice in an island setting (Gierus et al. 2022). In practice, this strategy would require rearing, transport, and release of potentially hundreds of such GM mice on an island to eliminate an invasive pest population (Campbell et al. 2019). Current modeling suggests successful eradication using this technology would require 25-30 years (Gierus et al. 2022, Combs et al. *in review*).

Prior to any field trials, GM lab mice must be tested under controlled conditions to ensure efficient transfer of

the genome edit to the target wild population. Currently, a major gap for the development of safe and effective application of genetic biocontrol is an understanding of how they might spread in real populations when the target species' spatial and reproductive ecology is considered. Controlled pen trials with GM and wild type mice assessing rodent behaviors and reproduction, coupled with population modeling, will help fill this gap and better forecast efficacy for eradication of an invasive rodent population on an island.

To safely complete future GM rodent studies at the USDA National Wildlife Research Center (NWRC) (Fort Collins, Colorado, USA) that involve gene drive mice or another form of a genetic biocontrol rodent, we must have high confidence in mouse containment in our animal buildings, rooms, and arenas where free-ranging trials will occur. Therefore, our objective of this study was to design and test multiple containment strategies to ensure that free ranging wild (and non-GM) house mice remain contained in NWRC animal rooms. Our trials will inform the best materials and strategies to minimize risk of inadvertent environmental release of GM mice. For such trials and future studies, we envision a primary containment arena (walled area within an animal-secure room) that has a secondary containment barrier (e.g., band of live- and/or lethal-traps), within a "mouse-proof" animal room (i.e., the 3rd layer of containment). Below are the descriptions of our studies, including detailed methods, results, and interpretations.

METHODS

Mouse Capture and Preparation for Trials

House mice were live-trapped on livestock farms in northern Colorado (Fort Collins and Wellington). Mice were initially held individually, in numbered "shoebox cages" (i.e., plastic bins with a stainless-steel wire-lid, dimensions: 29 cm × 19 cm × 13 cm (l × w × h)) in NWRC animal rooms. During a 1-week quarantine period and when mice were being held for subsequent trials, mice were maintained on a diet of standard rodent chow pellets (i.e., Laboratory Rodent Diet, www.labdiet.com), occasional apple slices, and water was always available. Each shoebox cage contained loose corn cob to line the floor, a plastic den tube, and cotton balls for bedding. Following quarantine, all mice received a radio-frequency identification (RFID) tag (AVID Company) that was inserted between the shoulder blades with an RFID tag applicator. To safely insert the RFIDs, mice were sedated using isoflurane gas. All animal uses for this study were approved by USDA/NWRC's Institute for Animal Care and Use Committee, under NWRC protocol QA-2861.

There were n=75 mice of approximately equal sex ratio used in this study, all of which were adults when trialed, and weights ranged from 10 g to 25 g. There were three types of trials conducted, each with varying sizes of containment units made of 0.65 cm thick plastic: 1) Clear-box (15 gallon "plexiglass" aquaria) trials where each containment unit was 76 cm × 46 cm × 46 cm (l × w × h), 0.35 m^2, and made of clear polycarbonate sheeting (Lexan brand) (Figure 1), 2) White-box trials were crafted in dimensions of 60 cm × 60 cm × 60 cm (l × w × h), 0.36 m^2, and made of white/opaque polyethylene sheeting (Figure 2), and 3)

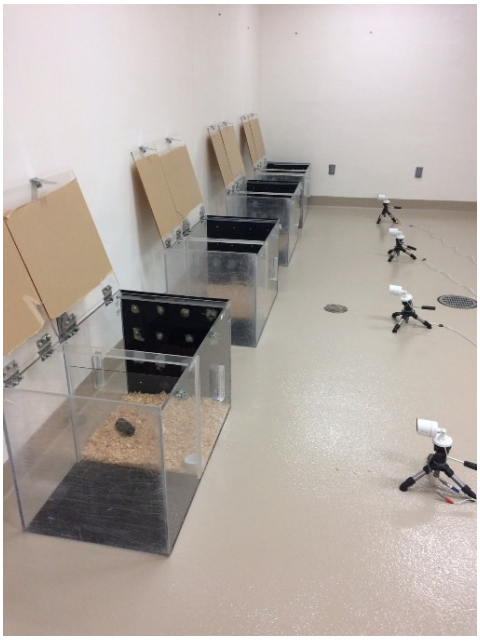

Figure 1. Picture of 4 of the 8 clear box trial containment units (lids remained open during trials) with monitoring cameras. Note the clear plastic partition that divides each unit where food and mate incentives were placed in attempt to encourage the mouse to escape to the other side of the partition.

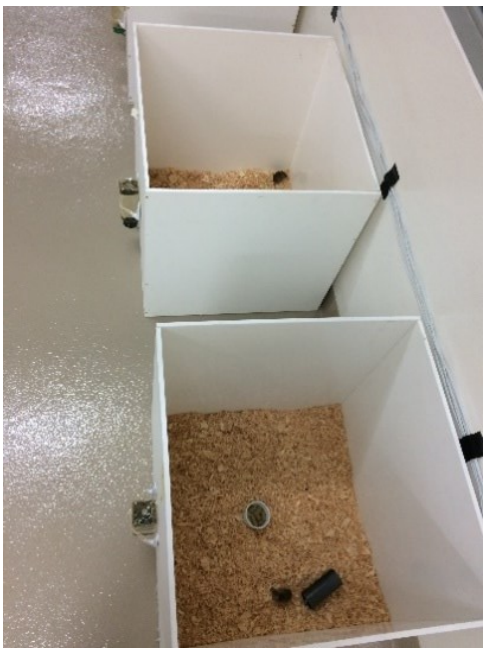

Figure 2. Picture of 2 of the 5 white box trial containment units. Monitoring cameras were used to document mouse behaviors and escape attempts. After completion of the white box trials without mouse escapes, the white box materials were used to build the final containment unit, the arena (Figure 3).

Arena trials, where a single containment unit of dimensions 4.9 m × 4.9 m × 0.9 m (l × w × h), 24 m^2, was made of the same white/opaque polyethylene material used in

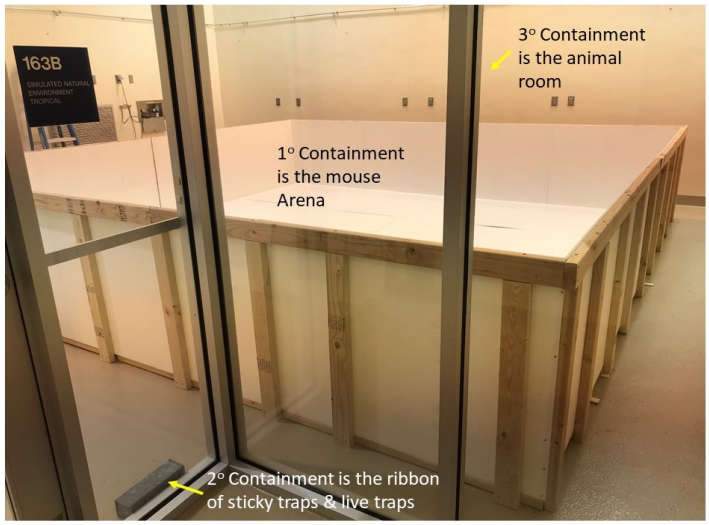

Figure 3. Picture of the 3-layer (1° = primary layer, 2° = secondary layer, 3° = tertiary layer) house mouse containment strategy used and tested at NWRC: a 4.9 m × 4.9 m × 0.9 m (l × w × h) mouse arena (still being built in this picture) (primary containment unit), and the other two levels of containment shown in a Simulated Natural Environment (SNE) animal room.

white-box trials (Figure 3). The arena was placed in a 73 m^2 animal room, referred to as a Simulated Natural Environment (SNE) room, with full climate and photoperiod control as well as adjoining observation rooms with space for computer monitoring equipment. Mice that were in holding cages and in trials were checked daily to ensure health and availability of food and water. All animal rooms in the BSL2 facility were maintained on a 12-hr light and 12-hr dark cycle.

House Mouse Containment Trials: Boxes

The objective of using the box trials was to determine if the clear or opaque materials would be appropriate materials to contain wild house mice in a larger arena setting, and to determine the minimum height of walls for mouse containment. For trials with both box types, the top of the box was open and the following items were placed in each box with the mouse: wood shavings covered the floor to 1-2 cm depth, a PVC-tube hide, two cotton balls for bedding, a sipper water bottle, and a ceramic food dish. All box trials occurred in animal room ISRB 153, where temperatures were 19-23°C and average relative humidity was approximately 30%.

Clear Boxes

The clear boxes were equipped with a 3.2 mm thick plexiglass/acrylic partition separating the box into two quadrants. The partition, which was secured in a plexiglass track that had a 4.8 mm groove, allowed a test animal on one side of the partition to be able to see incentive items (e.g., open space, food items, individual of opposite sex) on the other side of partition during incentive trials.

There were eight clear boxes used for each trial (Figure 1), and there was one mouse placed in each box, except in one trial where a second mouse of opposite sex was placed on the opposite side of the partition. Once a single mouse was placed in each box, the following sequence of incentive trials occurred and mice were observed daily to determine if escape from the boxes had occurred: 1) for the first 7-10 days, all mice (n=8) received no additional incentive items for escape, 2) incentive trial 1, 72 hours: n=4 treatment mice received peanut butter incentive, where 10 g of Skippy Creamy peanut butter was spread on the top 3 cm of the walls of the box and at the corners; the other n=4 mice received no peanut butter incentive, 3) incentive trial 2, 72 hours: the same n=4 treatment mice received a food buffet, including three shelled peanuts, a quarter-sized ball of peanut butter mixed with oats, and an apple slice, placed on the opposite side of the clear partition; the other n=4 mice received no food buffet, 4) incentive trial 3, 5 days: the same n=4 treatment mice received a food buffet on the opposite side of partition; n=4 mice with no food buffet on other side of partition; and food was withheld from all 8 mice for approximately 16 hours per day, and 5) incentive trial 4, 7 days: the same n=4 treatment mice received an individual of the opposite sex on the other side of the partition, n=4 mice did not receive another mouse on the other side of partition.

In all clear box incentive trials, the mice receiving treatment and those in the control group were randomly assigned. Three sets of the n=8 clear box incentive trials were completed in 2018 (2/20-3/26; 3/27-4/23; 5/14-6/11), using a total of 36 mice (i.e., 24 mice used for incentive trials 1-4, and an additional 12 mice of opposite sex were also used during incentive trial 4). A Swann security surveillance cameras system (DVR8-4500 8 channel 1080p digital recorder, with 1080p and 720p HD cameras) was used where cameras were positioned to observe each house mouse in its containment box, and the videos were retrieved and analyzed if there was evidence of a breach, and a subset were viewed to confirm if mice had breached the barrier and returned to their placed containment position.

White Boxes

There were five white boxes available (Figure 2), and a single mouse was placed in each box and monitored for

escape for 7 days. The dates of these trials (n=5 for each) in 2018 were: white box trial #1: 3/19-3/26; white box trial #2: 3/26-4/2; white box trial #3: 4/2-4/9; white box trial #4: 4/9-4/16; white box trial #5: 4/16-4/23; white box trial #6: 5/14-5/21; white box trial #7: 5/21-5/29; white box trial #8: 5/29-6/5. One mouse was trialed twice, so there was a total of 39 mice used in the white box trials. Cameras were placed to monitor each mouse when on trial with methodology as described above.

Mouse Containment Trials: Arena

Primary Containment

Following the box trials, a 4.9 m × 4.9 m × 0.9 m (l × w × h) arena was constructed with a frame of wood 2×4's and polyethylene (white/opaque) sheeting, which was the same polyethylene successfully used for mouse containment in the white box trials. Because rodents are notorious for excavating into materials that are not smooth, all screws and seams (where the sheeting joined) on the interior walls of the arena were covered by gluing, with construction glue, stainless steel sheet-metal and strips of the same polyethylene (Figure 3). The arena was constructed within one of NWRC's SNE animal rooms (room ISRB 163B). These animal rooms give us the options of simulating any biome or habitat through sophisticated climate control (air temperature and humidity, precipitation, light cycle, and full solar wavelength spectrum) and substrate control (e.g., soil, rock, live plants). The arena was equipped with 2-4 feeding stations that were secured to the arena floor and AVID pit-tag readers were positioned beneath the floor and feeding stations (thus outside the arena) so all mice visiting the feeding station would be identified and automatically recorded with the date and time of visitation. Placement of the pit-tag reader outside the arena allowed the arena to remain free of wires, power cords, and additional devices that could be chewed and damaged by the mice. The arena floor was covered by 2-5 cm of wood chips. The feeding stations contained standard rodent chow pellets and water (in sipper bottles) in excess, and apples wedges were offered weekly. PVC-tube hides were available, and there were always more black plastic den boxes present than there were individual mice. Cotton balls were placed in each den box to allow for nesting. Monitoring cameras (one per corner of arena) were continuously active (day and night) and footage was reviewed when breaches occurred or otherwise as necessary.

During June-December 2018, and at any one time, six to 26 mice (range: 12-25 g body weight) were placed in the arena and simultaneously monitored for their ability to escape from the arena. All mice were previously used in the box trials. We altered mouse density to simulate conditions that may encourage escape (e.g., few individuals may be lonely and feel motivated to escape the arena; alternatively, too dense may facilitate escape). We added potted plants, leaf litter, and sticks to simulate natural conditions, and we altered the temperature and humidity to simulate both temperate (19-23°C, ~30% average humidity) and tropical (28-34°C; ~70% average humidity) ecosystems and to ensure arena materials could adequately withstand these microclimate conditions. Evidence of fighting, injury, and death were noted when observed; evidence of gnawing on the containment material was also noted. Because all trials typically included an equal male-female ratio, breeding and pup rearing were observed behaviors. At the end of the trials, all individual house mice were euthanized and had tissue preserved for future parentage and mate competition studies.

Secondary Containment

Eight unbaited Sherman live traps (2 per corner) were positioned along the walls of the animal room. These traps surrounded the arena, comprising our secondary containment layer for house mice at NWRC, so if there was a breach from the arena then mice could remain contained within the room in traps (Figure 3).

Tertiary Containment

The animal room (generally at least 8 m × 4 m, and ISRB 163B with the arena was 73 m^2) was the third containment layer for house mouse containment at NWRC (Figure 3). To ensure mouse containment within these animal rooms, all surfaces (e.g., walls, floor, drains, vents) were inspected and made "mouse proof", as best as possible, by covering caulked seams, holes, and gaps with metal. Additionally, the entrance of the room was equipped with a double-door anteroom.

Observations

All mice were physically checked by staff a minimum of daily during all trials, and included checks of RFID tag readers. The RFID tag system enabled us to ensure each mouse had "checked in" (visited the food and water station where the RFID tag reader was mounted beneath) each day. Den boxes were checked at least weekly by opening them to determine if pups had been born. The potential parents (at least the mothers) were identified using a handheld RFID tag reader that would read through the plastic nest box; this enabled low disturbance to the pregnant or nursing mothers. The suspected mother with her dependent pups were removed from the arena to either euthanize the pups or individually cage the mother with her pups to complete weaning. The live-traps (i.e., containment layer #2) were checked daily and if a mouse was observed its RFID tag was read to identify if it was from within the arena.

Statistical Analysis

For clear box trials, chi-square tests were conducted to compare evidence of breaches between treatment (n = 12) and control (n=12) for each trial. The chi-square tests were conducted in *R* (version 3.4.1). Because the arena was not replicated, we provided summary statistics but no additional statistical analysis for these trials and data.

RESULTS

Mouse Containment Trials: Boxes

During the 110 days (or 2,640 hours) of monitoring of 36 house mice in clear (plexiglass) boxes, there were two mice during the first set of trials that escaped from their boxes but were contained in the room. Details of each of these breaches are outlined below. Once the situations that facilitated the breach by the two mice were fixed, there were no further breaches or escapes during the > 70 days more of monitoring with the clear box trials. Extra incentives of food and individuals of the opposite sex available

across the partition, which would have been detectable by sight and smell, did not influence the likelihood of mouse escape ($P > 0.999$; $\chi^2 = 0$; df =1 for each food incentive comparison), including the two incidents when a mouse successfully breached by chewing through the plastic partition ($P = 0.4602$; $\chi^2 = 0.54$; df =1). Further results and details of the clear box trials are described below and in Shiels (2022).

During the clear box trials, set #1, there were two mice (RE32 on 3/6/2018, and RE08 on 3/8/2018; both were males) that chewed through the 3.2 mm partition (acrylic /plexiglass) and got to the other side of the cage. These two mice were not treatment mice, and therefore did not have peanut butter or food buffet incentives. The size opening at the widest points that each chewed was the same for the two mice (1.59 cm × 2.22 cm). The boxes where RE32 and RE08 resided were different than the other six boxes because the tracks that held the plexiglass in place did not run to the bottom of the box; this allowed a small gap (~1.6 mm) to be exposed between the wall of the box and the partition, and the gap was apparently large enough for each mouse to get their teeth into it so a hole could be chewed in the partition. On 3/9/2018, the same two mice (RE32 and RE08) escaped from the clear boxes upon the partitions in each box being completely removed from the boxes by our staff. Video surveillance showed that these two mice quickly crawled up the track that the plexiglass partition had been in within 4 minutes of when staff removed the partitions temporarily. The additional clear box trials (i.e., sets 2 and 3) had no breaches or escapes, and further details can be found in Shiels (2022).

There were no escapes during the white box trials during a total of n=39 mice monitored (3/19-6/5, 2018) for 57 days (or 1,368 hours). Therefore, we decided that the opaque material used in the white box trials would be best as the test material in the arena setting.

Mouse Containment Trials: Arena

We decided on arena walls of 91 cm tall to be conservative (ensure containment), and to account for the 35 cm heights that we noticed house mice would jump during box trials. The 35 cm heights jumped was mostly from a standing position, and therefore we predicted that greater heights may be achieved by mice running, jumping, and launching off objects placed within the arena setting such as den boxes, feeding stations, and potted plants.

During the 6-month long arena trials, the number of mice in the arena at once (intentionally placed) ranged from six to 26. Specifically, six mice were added on 6/11/2018, yet 1 week later one died, and three more were added (total was eight mice on 6/18/2018). One mouse died from aggression on 6/21/2018, and on 6/25/2018 four more were added (total was 11 mice on 6/25/2018). On 7/3/2018, one mouse was wounded and removed leaving 10 total mice in the arena. On 7/11/2018 while weighing 12 additional mice that were about to be added to the arena, two escaped during weighing, which was done in the room with the arena but just outside of the arena. These two mice were caught the next day in the secondary containment layer (Sherman traps). Thus, there were 22 mice present in the arena on 7/12/2018. On 7/16/2018 there were four more adult mice added to arena to even out the sex ratio, so there were 13 males and 13 females. On 7/24/2018 two mice died of natural causes (confirmed by veterinarian necropsy), resulting in 24 mice in total in the arena.

On 7/27/2018, one mouse escaped the arena (RE48, male), which had been added 11 days prior. It was found in a live trap (secondary containment layer) in the NE corner of the room. This was 46 days after we began housing mice in the arena. After putting cameras on all walls of the arena, and putting that male back into the arena, we could not replicate the escape. Our staff went through the entire arena looking for possible ways out, and they found none. We concluded the most plausible mechanism of escape was on the previous day when the staff cleaned out all the wood shavings, there must have been a mouse in the shavings that was deposited into the trash bin (which remained in the room). The mouse then climbed out of the trash and was caught in the live trap. No other mice escaped the arena during the duration of the study, which lasted 4 months longer.

DISCUSSION

Through rigorous testing of containment materials and strategies using 75 wild-caught house mice that were observed for >4,000 hours, we conclude that we can contain and otherwise prevent escape of house mice from the NWRC animal rooms using the containment materials and protocols outlined in this study. The polyethylene material that was tested on small scale white box trials, and then subsequently tested in the arena, showed 100% containment of house mice during their natural activities. However, the one breach of a single mouse from the arena highlights that humans may be a cause of a mouse escape. Therefore, strict staff training and standard operating procedures (e.g., sifting of wood chips prior to disposal) will likely be necessary to maintain highly biosecure facilities.

Despite the very low probability that house mice would be able to breach all three containment levels before being detected or captured, 15 radio-collared mice that were released outside the animal research buildings (data not shown, but see Shiels 2022) has given additional information that can be applied to improve the biosecurity plan implemented for the facility's surroundings during periods when rodents are on site for research. Based on our radio tracking results, there is as little as a 1.5-day window to recapture or kill a mouse that had escaped the building before it exits NWRC campus. Recapture and elimination strategies and devices should concentrate in a 40 m band around the buildings, but some efforts should span as far as 100 m from the buildings, and bait stations that are currently in place around each NWRC building should be maintained and potentially expanded when GM rodent trials are occurring at NWRC (Shiels 2022).

Genetic biocontrol population specific constructs are being explored for preventing gene drive from affecting nontarget wild populations (Sudweeks et al. 2019). Oh et al. (2022) scanned wild mouse genomes and identified genetic sequences that were fixed in introduced island populations of house mice, but harbored potential resistance alleles in nontarget populations (e.g., continental house mice). Such technology and genetic specificity could be used as an additional biosafety tool in free-ranging labor-

atory trials. The NWRC physical containment trials presented here and the radio-tagging trials where 15 (non-GM) mice were purposefully released outside the NWRC building to determine behaviors and key response times (Shiels 2022) have prepared us more fully for future GM mouse trials and have helped to optimize the facility's biosecurity plans so such work can occur safely and efficiently in the future.

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