Refinements for Rodents and Rabbits in Research Institutions

Making Life Easier for Animals in Laboratories

BY I. JOANNA MAKOWSKA & ANNA S. RATUSKI

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In the words of the wonderful Jane Goodall (1934–2025), who inspired us immensely in our young years:

"You cannot get through a single day without having an impact on the world around you. What you do makes a difference, and you have to decide what kind of difference you want to make."

We dedicate this book to the people who care for animals in laboratories with compassion, and hope that it inspires them to make a difference in the lives of every mouse, rat, chinchilla, gerbil, guinea pig, hamster, mole rat, vole, and rabbit who depends on them.

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Refinements for Rodents and Rabbits in Research Institutions: Making Life Easier for Animals in Laboratories

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INTRODUCTION

It is easy to overlook the needs of small rodents and rabbits. In the laboratory, they tend to draw less attention to themselves than many other species: They are usually asleep during the day (most are nocturnal or crepuscular); they rarely emit vocalizations audible to the human ear (rodents communicate in the ultrasonic range); and they tend to hide any discomfort they are experiencing. Rodents, especially, are small and are usually present in very large numbers, which challenges our ability to connect with any one of them on a more personal level. In popular culture, rodents and rabbits are commonly portrayed as unwanted "pests" or as easy, low maintenance "starter" pets for children.

Yet, those of us who have spent time in the company of small rodents or rabbits have discovered that they are complex, curious individuals, each with a unique personality. Given the chance to express themselves, they are active and playful. They seek out opportunities to explore, forage, and build comfortable homes. With the necessary care and attention, they seek out and enjoy human affection.

This book summarizes 15 years of scientific literature on refinements to the care and use of rodents and rabbits in research. The goal is to synthesize the knowledge gained from these studies into a user-friendly resource on how to safeguard and promote the welfare of rodents and rabbits in experimentation. The book is organized into seven chapters, each on a different topic related to the care and use of these animals in research. Each chapter begins with a description of the topic and why it is important for both animal welfare and research outcomes. The bulk of each chapter then summarizes the scientific literature regarding this topic, organized by species (mice, rats, other rodents, and rabbits). A "Key Takeaways" summary box is provided at the end of each chapter for quick reference.

The book builds upon a previous volume, *Variables, Refinement and Environmental Enrichment for Rodents and Rabbits Kept in Research Institutions: Making Life Easier for Animals in Laboratories*, published by the Animal Welfare Institute (AWI) in 2006. While the primary focus here is on literature published since the year 2010, older studies are described whenever they are needed for context.

As with the previous volume, this book relies primarily on peer-reviewed scientific literature, but also brings in other evidence—articles published in professional magazines such as *Laboratory Animal Science Professional* and *Animal Technology and Welfare*, for example—where it has important practical relevance. It also includes insights from members of AWI's Laboratory Animal Refinement and Enrichment Forum (LAREF), where individuals who care for animals in laboratories discuss and share first-hand experiences, innovations, and insights regarding ways to improve living conditions for the animals.

In sum, the book references 471 articles that address issues related to the following species: mice (202), rats (127), rabbits (49), rodents and rabbits, general (29), all species, general (23), hamsters (10), guinea pigs (9), mole rats (8), chinchillas (6), voles (4), gerbils (3), and degus (1).

All articles referenced herein are searchable via AWI's online Refinement Database, a curated collection of scientific articles and books on topics related to the refinement of housing, husbandry, care, and use of animals in research and testing. Database searches can be filtered by **Animal Type** (this includes all taxa and dozens of species) and **Topic** (all those covered in this book, plus more).

We are very grateful to Dr. Maisy Englund, Dr. Andrew Fenton, Dr. Melanie Graham, and Cathy Liss for their thoughtful contributions and valuable insights at various stages of writing this book.

AWI'S POSITION ON RESEARCH USING ANIMALS

Providing animals in research with the highest available standards of care should be a prerequisite for their use (Makowska & Weary, 2019). This is not only in line with societal expectations but also likely to render the results of the research more generalizable and repeatable (Garner, 2005; Richter et al., 2009). Moreover, providing high levels of care is associated with greater job satisfaction and lower compassion fatigue for animal caretakers (LaFollette et al., 2020).

AWI's *Position Statement on Research and Testing Using Animals* is as follows:

Research must not be conducted on animals unless, at minimum, the research is planned and executed in accordance with the principles of the "3Rs" (replacement, reduction, refinement) outlined by Russell and Burch (1959) and includes the following standards:

1. The animals must be maintained in an optimum, species-appropriate environment.
2. The animals must be under the care of professionally trained, compassionate personnel.
3. The animals' pain, discomfort, fear, and distress must be prevented or at least minimized by considerate and scientifically sound experimental design and appropriate use of anesthetic, analgesic, and tranquilizing drugs.

All the studies in this book provide information about refinement. We recognize that in some of these studies, causing harm to the animals involved was an inherent aspect of the research topic—for example, when animals were used to study therapies for specific diseases. In a few cases, however, some of the research questions could have been addressed using less harmful methods or in a manner that otherwise better prioritized animal welfare—for example, by using a sucrose preference test or an affective bias test rather than the forced swim test (see Animals in Science Committee, 2023) to measure depression or anhedonia. Studies using methods that fail to minimize pain, discomfort, fear, or distress do not meet the standards articulated in AWI's Position Statement. Throughout this book, these studies are flagged with an asterisk (*) and a footnote to acknowledge that the methodologies are deemed to have caused undue harm or suffering in addressing the research question. Such studies are included in the book when their results—notwithstanding how they were obtained—corroborate or stand alone in providing important welfare information, with implications that may support tangible improvements for future animals used in research.

Most studies within this book describe animals housed within small and relatively barren cages. These cages are not "optimum, species-appropriate" environments and therefore do not meet the standards articulated in AWI's *Position Statement*. Unfortunately, the use of these cages continues to be standard practice at the vast majority of research facilities, and individual researchers generally have little or no influence over this choice (in contrast to their ability to select the research methods used in their studies). For these reasons, the use of these cages is not flagged in this book.

Housing

What Is It, And Why Does It Matter?

Confinement to a cage or pen is arguably one of the most significant stressors for animals in laboratories, because it restricts their freedom and isolates them from their wider surroundings. Indeed, the researchers who established the first colony of laboratory rats over a century ago observed that "confining a rat to the limited quarters of a cage necessarily restricts its activities, modifies its mental processes, and influences its growth and development" (Greenman & Duhring, 1923). Because rodents and rabbits in laboratories spend the bulk of their lives within the confines of their cages or pens, the size and features of these dwellings largely set the limits of their life experiences and impact them in profound ways (Lewejohann et al., 2020).

The conventional "shoebox" cages used for rats and mice are over 250,000 times smaller than the animals' natural home range (Lahvis, 2017). This cage was originally created in the early 1900s and was primarily designed for convenience of handling and cleaning (Eaton & Cabell, 1961; Greenman & Duhring, 1923; Scharmann, 1991) rather than animal welfare. The conventional cage has changed little over the decades; indeed, today's design is based more on tradition than scientific evidence for what is ethologically appropriate (Gaskill & Pritchett-Corning, 2015; Greenman & Duhring, 1923; Mieske et al., 2021). In recent decades, the growing field of laboratory animal welfare science has led to a greater call for cage and pen designs that are more species-relevant and do a better job of addressing the animals' physical and mental needs.

While a "good life" may not be feasible in conventional rodent and rabbit cages, some features can be improved to provide better welfare—for example, by supplying appropriate bedding and furnishings, and affording animals some choice and control. Ultimately, the aim should be to move away from small, barren cages to less restrictive options. A clear roadmap for how to achieve this for rats and mice is provided by Makowska and Weary (2019).

Summaries of Current Refinement Research

Mice

In the last two decades, individually ventilated cages (IVCs) have become increasingly popular because of the advantages they present for human and animal health. In an IVC system, each cage constitutes a self-contained microenvironment, which minimizes the spread of allergens or pathogens between cages and between animals and humans. However, the persistent draft and vibration associated with ventilated caging introduce new challenges for animal welfare.

Each IVC system has a unique design and differs in the number of air changes per hour (ACH), the locations of the air inlet and outlet (e.g., at the lid or near the cage floor), and general cage configuration (e.g., cage shape, location of the food hopper and water). A number of research papers have investigated the characteristics that contribute to IVC-associated stress and proposed ways to address these.
In general, mice do not like drafts. Baumans et al. (2002) found that female BALB/c mice preferred static cages over ventilated cages. However, this preference disappeared if mice were given nesting material to shield from the draft. Similarly, Krohn and Hansen (2010) found that, if air was delivered at a constant rate of 1.6 feet/second at animal level, Sca:NMRI female mice did not discriminate between cages with 40, 80, or 120 ACH. In contrast, when the air change rate was held constant at a low 40 ACH, the mice preferred cages with air speed rates of 1.6 feet/second over those with rates of 3.3 feet/second.

The location of air delivery also affects how the circulating air is perceived by mice. Burman et al. (2014) housed female mice of two strains (C57BL/6J and BALB/c) in two types of IVCs: One delivered air at 75 ACH at the *cage lid* level and the other delivered air at 50 ACH at the *animal* level. Mice of both strains showed higher anxiety in elevated plus maze and open field tests in the cages that delivered air at the animal level, even though the air change rate in those cages was lower. Furthermore, these mice ate more during the first week and drank more water throughout the study, but there were no differences in body weight. Finally, these mice had higher "bedding pushing" scores, where they pushed bedding to one side of the cage so that it covered the air inlet. In a different study, female C57BL6/JArc mice in cages furnished with an igloo-shaped shelter, tissues as nesting material,

an air inlet at the lid level, and an air outlet at the animal level (90–120 ACH with air speed of 0.39 f/s) exhibited more anxiety in the elevated plus maze test compared to mice in static cages (Logge et al., 2013).

Spangenberg et al. (2014) investigated maternal behavior and pup development in C57BL/6NCrl and Crl:NMRI *Foxn1*^{nu} mice housed in three types of IVCs with crinkle paper as nesting material. They found that the features of each cage system, such as the location of air inlets and outlets, affected maternal performance and pup development. For example, lactating C57BL/6NCrl females built higher quality nests—suggesting greater need for insulation—in a system that had the air inlet at the animal level and an air change rate of 50 ACH over systems with the air inlet at the lid and either lower (40 ACH) or higher (75 ACH) air change rates. Moreover, hairless Crl:NMRI *Foxn1*^{nu} females and their pups had lowest weights in the system with the air inlet at the animal level.

Mice likely avoid drafts because they cause cold stress. David et al. (2013) showed that male Prdkc^{scid} and Nu-*Foxn1*^{nu} mice housed without a shelter in IVC cages at 60 ACH had histologic signs of cold stress, higher nonshivering thermogenesis, and larger adrenal glands compared to mice housed in static cages. (Larger adrenal glands can be caused by cold or psychological stress.) Adding an igloo-shaped shelter to the IVC improved, but did not eliminate, these signs of stress. Pasquarelli et al. (2017) found that male C57BL/6JRj mice transferred to an IVC had lower body weight 3, 13, and 23 days after the transfer compared to mice who remained in static cages, even though both cage types contained an igloo-shaped shelter and cellulose tissue as nesting material. Wang et al. (2019) found that female ICR mice housed in IVCs at 80 ACH without a shelter had lower immune cell counts compared to mice housed in static cages. (The immune system can be compromised by cold and/or psychological stress.)

Stover and Villano (2022) compared breeding performance and cage cleanliness in three IVC systems: Allentown 's NexGen MAX, Animal Care Systems' Optimice, and Tecniplast's Emerald Rack, High Density EMM096X. Breeding trios of Swiss Webster or BALB/c mice were housed in these cages with corncob bedding and one cotton square; two generations of these mice were monitored for three breeding cycles each. On most of the outcome variables tested, no differences were observed between the three caging systems. Swiss Webster litter sizes at weaning, however, were smaller in the Allentown cages compared to the other two cage types. Overall, the percentage of pups surviving to weaning didn't differ between systems. However, the difference in survival rates between BALB/c pups and Swiss Webster pups was most pronounced in

the Allentown system (96.8% vs. 70.6%, respectively). Microenvironmental parameters didn't differ between caging systems over the study period, but there was a trend for the Allentown cages to be cooler and more variable than cages in the other two systems; also, Allentown cages were significantly cooler than the other two on day 8 of the study: 69.2 °F (20.7 °C) versus 74.4 °F (23.6 °C) and 73.4 °F (23 °C).

One way to allow mice to better thermoregulate is to provide a heated floor space in their cage rather than heating the entire room (which would be uncomfortable for humans) or heating the air within each cage (which would be inefficient). Gordon et al. (2017) described how to make an inexpensive "heater" using a hand warmer, an aluminum plate, and a cellulose sponge. The device can be placed under the mouse hut, keeping the temperature inside the hut at a steady 86–90 °F (30–32 °C). Mice can move to other locations in the cage to access cooler temperatures, thus giving them some behavioral control over their environment (Gaskill & Garner, 2017). For more information on mouse thermoregulation, see chapter on **Environmental Enrichment**.

Bailoo et al. (2018) tested how space allowance affects certain measures related to mouse welfare by housing male and female C57BL/6ByJ and BALB/cByJ mice in various cage and group-size combinations. Three types of cages were used: Makrolon Type II (10.5 x 8 x 5.5 in.), Makrolon Type III (17 x 10.5 x 6 in.), and a cage conforming to the guidelines for housing pet mice in Switzerland (24 x 16 x 5.5 in.). All were furnished with wood shavings to a depth of 0.75 inches and with 10–11 grams of shredded paper for nesting material. Within each cage type, mice were housed in groups of three, five, or eight individuals. Over the course of 10 weeks, there were no differences between any treatments for body mass, fecal corticosteroid, behavior in the open field test, recurrent perseveration in a two-choice guessing task, home-cage activity, or stereotypic behavior. The only difference was an increase in aggression in male BALB/cByJ mice with increasing group size, which led to attrition; cages with more attrition also had lower food and water intake. The authors concluded that space allowance has little impact on mice within the range of conditions commonly found in laboratories, except for increased aggression in some strains. In a review paper, Whittaker et al. (2012) explained that mice in captivity may not have a specific "spatial need" but do have a "behavioral need" that requires a certain amount and quality of space. Because mice are thigmotaxic (they like to maintain contact with a vertical surface), what matters is not only the floor area but also perimeter length, ratio of perimeter length to floor area, ratio of length to width, and position of resources within the environment. The review concluded that the available scientific evidence suggests that the quality of the environment is much more important than its size, provided that the combination allows the expression of the animals' full behavioral repertoire.

Figure 1: In Slater and Cao (2015), groups of 10–20 mice housed within large, structurally complex bins had less fat, stronger immune systems, and lower serum corticosterone after just 5 weeks.

Indeed, Slater and Cao (2015) found that housing mice in large, well-resourced enclosures benefits their welfare. According to their published protocol, groups of 10–20 mice can be housed within large bins (47 x 35 x 30 in.) containing various resources—including wooden logs, tunnels, igloos with saucer wheels, and running wheels (Figure 1)—that are rearranged weekly. Food and water are provided from the wire lids of two conventional shoebox cages placed inside the large bin (a circular hole is drilled into one side of each standard cage to allow entry). Compared to male C57BL/6 mice living in groups of three to five within shoebox cages with a cotton Nestlet, those in the large and structurally complex environments had less fat, stronger immune systems, and lower serum corticosterone after just 5 weeks. Moreover, when the mice were injected with cancerous cells, tumors took longer to develop, and these tumors were smaller than those in conventionally housed mice (Cao et al., 2010).

A larger and more complex environment also enables mice to engage in the natural behavior of spatial segregation (i.e., designating different areas for different

activities). For example, Makowska et al. (2019) assessed how group-housed female Swiss Webster mice used space within a single conventional cage compared to a "complex system" that consisted of three interconnected conventional cages, one of which was red (Figure 2). Single cages were provisioned with nesting material (puck of compressed nesting material and cotton Nestlet), food, and water. In the complex system, all three cages were provisioned with nesting material, but only one cage contained food and water. The location of nests and latrines were scored at cage changing, once a week for 15 weeks. Mice in both systems segregated their nesting area from their designated urine area; indeed, nests and urine spots were in separate locations 98% of the time in both systems, with urine spots in the same location as food and water 98% of the time in both systems. Mice in the complex system typically carried all their nesting material into one cage to create a single large nest, while simultaneously carrying most of their bedding out of the nesting and neutral cages and into the food and water/latrine cage, perhaps as a way to dilute the scent of their waste products. The red cage was chosen as the nest cage 67% of the time, indicating a preference to nest in a darkened environment. In the complex system, feces were largely contained within the latrine cage. In the single cage, feces were present throughout the cage by the time locations were scored at cage changing; mice in this system may have also attempted to defecate in the same area where

Figure 2: Housing female mice within a system of three interconnected cages allows them to engage in spatial segregation that keeps clean and dirty areas separate (Makowska et al., 2019).

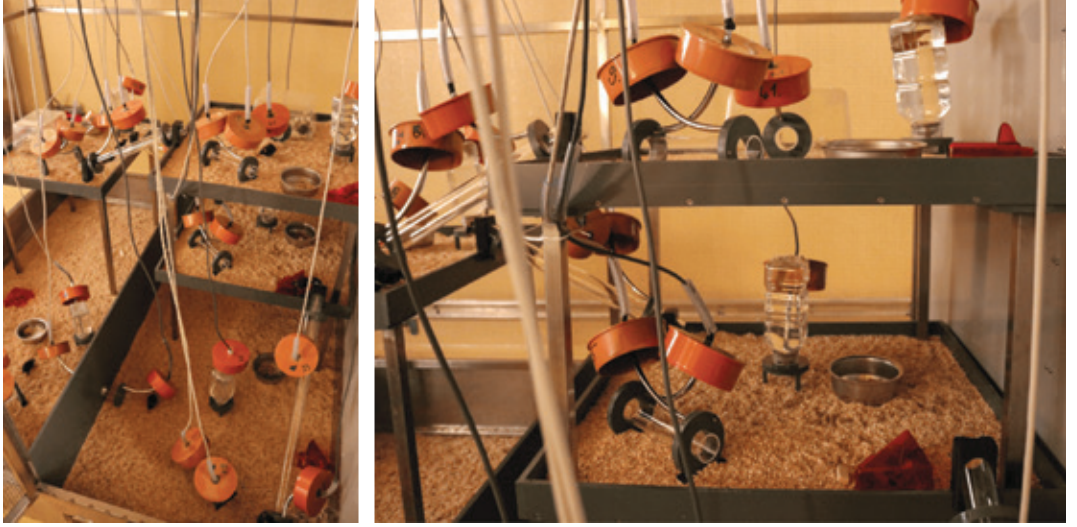

Figure 3: In Mieske et al. (2021), female mice living for approximately 2 years in a large wire-mesh enclosure with five distinct areas and three levels with deep bedding did not develop stereotypies.

they urinated but were unsuccessful in keeping waste segregated because, without physical boundaries, bedding moved and mixed during activity. Indeed, Boivin (2013) documented that female C57BL/6 mice group housed in shoebox cages lacked feces-free areas within 3 days.

Mieske et al. (2021) recorded individual mouse activity and behavior in a much larger, semi-naturalistic environment over the course of 11 months; mice had been housed in this environment from the age of 10 weeks, but data collection didn't start until they were 11 months old. The environment consisted of a large wire-mesh enclosure (67 x 67 x 83 in.) with five distinct areas and three levels connected with transparent plastic tubing (Figure 3). Each area contained aspen chip bedding to a depth of 1.2 inches, food, water, and a small shelter. Each of the two upper levels featured a nest box made from an inverted Makrolon Type II cage with holes drilled into it. The 20 female C57BL/6J mice living in this environment were each injected, under anesthesia, with a radiofrequency identification (RFID) tag between the shoulder blades so that their movements could be tracked using 27 RFID antennas placed throughout the enclosure. Their behavior was also scored via live observations. All mice followed similar patterns of behavior (highest activity at the beginning and the end of the dark period), but some individuals displayed consistently higher daily spatial activity levels. The most common behavior observed was social exploration (e.g., approaching and sniffing a cagemate), followed by maintenance behaviors (e.g., eating and drinking) and nonsocial exploration. Stereotypies were never observed. Overall, differences between individuals did not exceed those reported in published literature using conventional housing, which led the authors to conclude that even "quantum leap" improvements to animal housing do not inevitably lead to increased data variability.

Naturally, personality plays a role in how individuals perceive their living environment, and better housing conditions may benefit some individuals more than others. Sroka et al. (2024) characterized the exploratory personality of female C57BL/6 mice before housing them in simple or highly complex environments. The simple environment consisted of a shoebox cage with a hut, tunnel, cotton Nestlet, and wooden gnawing cube. The complex environment consisted of a shoebox cage placed within a large "playground" to which mice had permanent access via a hole drilled into the lid of the shoebox cage (Figure 4). The playground measured 20 x 12 x 20 inches and offered several levels and myriad resources that broadly fell into eight categories: rigid climbing

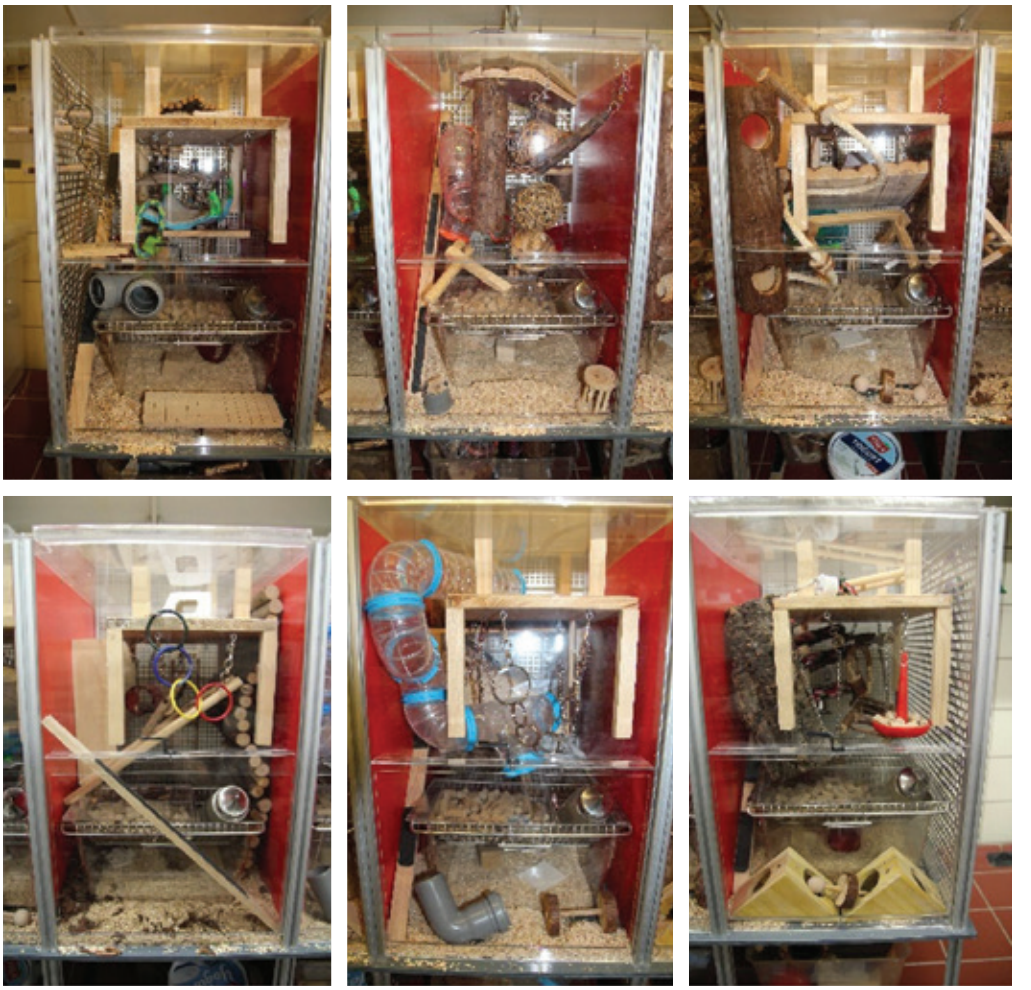

Figure 4: Compared to an enriched shoebox cage, complex housing consisting of a shoebox cage placed within a large "playground" better accommodated various mouse personality types and helped promote the welfare of all individuals (Sroka et al., 2024).

(scaffold, platform, ladder), flexible climbing (hammock, swing, seesaw), shelter (tunnel, hut, wooder bridge), structure (tunnel system, labyrinth), gnawing (wooden stick, wheeled dumbbell), extra bedding (digging tower, sand bowl, stone bowl), extra nesting, extra food (food hanger, food ball, food in maze). There were six playground versions, each with a different set of resources, and playgrounds were rotated on a weekly basis. Overall, for both highly exploratory and low exploratory mice, complex housing was associated with lower agonistic behavior and other signs of better welfare than simple housing. The effects, however, were more pronounced for highly exploratory individuals. For instance, while complex housing reduced stereotypies for low exploratory mice, it completely eliminated them for highly exploratory mice. On the reverse side, highly exploratory mice had higher levels of plasma corticosterone (indicative of stress) and fewer IL-6-producing myeloid cells (indicating lower immune functioning) in simple housing compared to complex housing. In contrast, levels of these stress and immune function indicators did not differ between housing systems for low exploratory mice. Thus, while complex housing benefits different personality types and can help to promote the welfare of all individuals within a population, the effects are especially pronounced for certain individuals.

Rats

The majority of conventional cages for rats are too short to allow adults to stand upright. This is deeply troubling, given that since its publication in 2011, the *Guide for the Care and Use of Laboratory Animals* has mandated that "at a minimum, animals **must** have enough space to express their **natural postures and postural adjustments** without touching the enclosure walls or **ceiling** [emphasis added]." Indeed, after surveying rat experts, half of whom had a background in laboratory research or veterinary school, a team of veterinary scientists developed a set of guidelines for appropriate pet rat housing and identified vertical space as one of 14 essential components (Neville et al., 2022). Specifically, "anything below 90–120 cm [35–47 in.] in height (at least three body lengths) is not suitable, and much larger cages are advised." They further advised that as much vertical space as possible should be provided over several tiers to allow rats to climb and rear. Horizontal space was also identified as an essential component; similarly to vertical space, it should be at least 35–47 inches or three body lengths wide, although as much horizontal space as possible should be provided. (Most of the other essential components pertained to cage contents.) While these guidelines were developed for pet rats, the authors stated that they could also be useful in the development of revised standards for rats in laboratories, since housing standards likely shouldn't differ depending on the purpose for which animals are kept.

Barker et al. (2017) examined the effects of group size and cage size on the affective state, cognitive ability, and social behavior of male Sprague Dawley rats. In one experiment, they compared housing two rats in smaller cages (14 x 8 x 9 in.) versus housing six rats in larger cages (24 x 15 x 10 in.); the resulting space per rat was similar in the two treatments. In another experiment, they compared housing two rats in the smaller versus the larger cages. There were no differences in the cognitive bias, open field, novel object recognition, or social interaction tests between treatments in either experiment. However, the authors found that within each experiment, dominant rats demonstrated more optimism and affiliative behaviors and lower anxiety than subordinate animals, suggesting that social rank is more important to rats' affective states than group size or space per rat. This study also suggests that, similarly to what was found for mice by Bailoo et al. (2018), space allowance in the absence of environmental complexity may have little impact on rats within the range of conditions commonly found in laboratories.

Wheeler et al. (2015) compared the welfare of male Sprague Dawley rats housed according to four treatments: in a conventional cage (control treatment; 22 x 13 x 8 in.); in a two-level cage (Tecniplast's Double Decker; 18 x 18 x 16 in.); in a two-level cage with access initially restricted to the bottom portion for about a month but opened up during testing; and in a two-level cage with initial access to the whole cage for about a month but restricted to the bottom portion during testing. Rats were single housed without enrichment and were limit-fed,* so results of this study reflect the behavior of rats who are under stress. Compared to rats in the control treatment, those who had been upgraded to full access were in a more positive affective state, and those who were downgraded to restricted access were in a more negative affective state in a cognitive bias test. Thus—again—within the range of conditions commonly found in laboratories, space allowance may not matter much *per se*, but an increase in space is perceived positively and a decrease in space is perceived negatively by rats.

Housing rats in two conventional cages connected by a short tube is a relatively simple way to provide them with more space and the ability to segregate their living quarters into functionally different areas. Amendola et al. (2023) group housed female Sprague Dawley rats in double-cage systems, where one cage contained food and water and both cages were furnished with a PVC tunnel and 15 grams of nesting materials (crinkle paper, cardboard cups, paper towel). Cages were weighed and photographed weekly for 5 weeks. The authors found that rats moved some of the

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** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

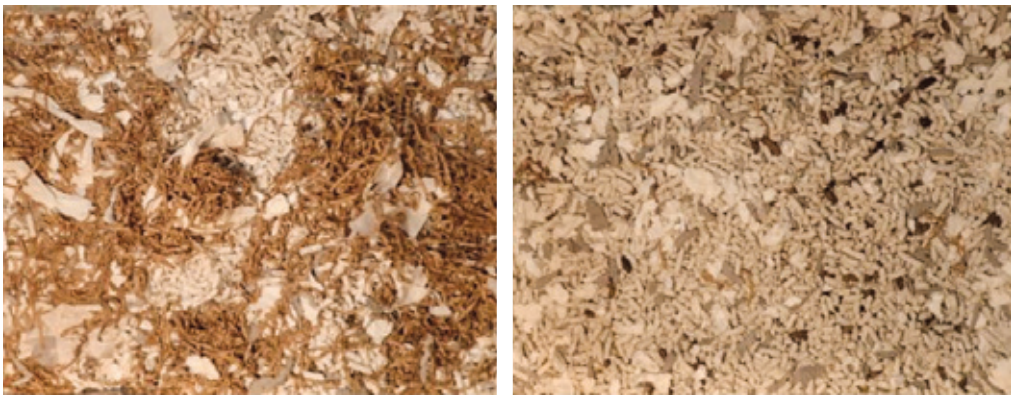

Figure 5: In Amendola et al. (2023), female rats housed within a double-cage system segregated their living space into clean and dirty areas by moving most of their nesting materials into one cage (left; no food or water access) and setting up a latrine in the other cage (right; food and water access).

nesting materials out of the food and water cage and used that cage for elimination. Indeed, both cages increased in weight, but the food and water cage increased by about twice as much as the other cage; in addition, the food and water cage had 9% less nesting material coverage compared to the other cage (coverage was estimated using image analysis software; Figure 5). Thus, rats segregated their living space into clean and dirty areas by placing a majority of their nesting material into one cage and setting up the latrine in the food and water cage.

Recognizing that welfare-friendly commercial laboratory cages for rats are not readily available, several researchers designed their own cages to compare them to conventional ones. Makowska and Weary (2016) housed female Sprague Dawley rats in groups of five within large, semi-naturalistic cages or in pairs within conventional cages. The semi-naturalistic cages consisted of a popular pet rat cage (MidWest Critter Nation double unit) measuring 36 x 25 x 49 inches and consisting of climbable horizontal wire bars and four levels with ramps. The bottom portion of the cage was filled with burrowing soil to a depth of 1 foot; the soil was contained within a custom-built Plexiglas box (Figure 6). The cage contained various environmental resources, including hammocks, a climbing structure, tunnels, paper towels, and foraging boxes. The conventional cages measured 18 x 9 x 8 inches and contained aspen chip bedding, a 4-inch-diameter, 7-inch-long PVC pipe, and two pieces of paper towel. The authors observed that rats in the semi-naturalistic environment regularly engaged in three behaviors that were not possible within the conventional cages: burrowing (i.e., burrow construction and maintenance), climbing, and upright standing. Rats

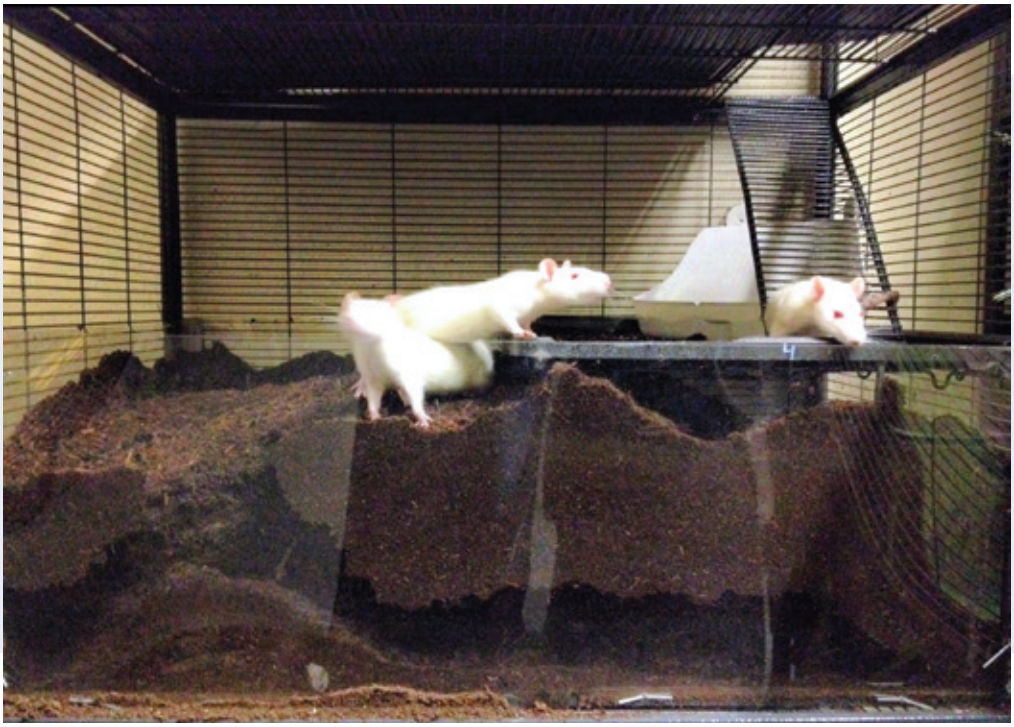

Figure 6: In Makowska and Weary (2016), burrow construction and maintenance emerged as a particularly important behavior to female rats housed with access to a foot-deep layer of soil.

burrowed an average of 30 times per day, and this rate remained stable as rats aged from 3 to 13 months old, suggesting that this behavior is particularly important to rats. Climbing was common when rats were 3 months old (75 times per day, sometimes using the ceiling as monkey bars) but declined as rats aged (25 and 6 times per day at 8 and 13 months old, respectively). The duration of each bout of climbing also decreased as rats got older; the authors speculated that climbing likely declined because of a natural loss in muscle strength and coordination as rats aged. Upright standing was the most frequent behavior of the three (180 and 75 times per day at 3 and 13 months old, respectively) and appeared to serve two distinct functions: exploration (head angled upward, with slight movements of the head as if the rat was sniffing) and upright stretching (back arched, head thrown back with a yawn, one foreleg outstretched). Because upright stretching is a natural postural adjustment in rats, it is imperative that they be housed in cages tall enough to accommodate this behavior. Indeed, the authors found that rats in semi-naturalistic environments performed two types of stretches: upright and lateral (similar to the "downward dog" yoga pose). In conventional cages, rats were not able to stretch upright, but they stretched laterally about eight times as much as rats in the semi-naturalistic

environment. Overall, the rate of stretching was six times higher in the conventional cages when both types of stretching (upright and lateral) were accounted for in semi-naturalistic environments, suggesting that conventional cages induce stiffness and positional stress that conventionally housed rats attempt to alleviate by frequent stretching. These findings showed that conventional laboratory cages interfere with important natural behaviors, and this is likely to compromise rat welfare.

Brenneis et al. (2017) developed a modular housing system from a commercially available ferret cage, allowing rats to be housed in large colonies of up to 48 individuals. The team successfully housed male Lister hooded rats from 6 weeks of age in a cage with approximately 24 square feet of floor space, with upper and lower levels connected by a custom external staircase and by holes that rats could jump through. The rats were injected subcutaneously with an RFID chip, and the cage was outfitted with RFID antennas to track their location, weight, and activity; these combined data were used to identify rats who may be ill. Anecdotally, this housing system was described as more ergonomic and requiring less time to transfer rats into a new cage during cleaning compared to conventional cages, because the rats were generally more cooperative and sociable. Similarly, Clarke and Ioannou (2018) developed a caging system to house rats in large social groups using existing multilevel cages for ferrets. Two cages were connected using a flexible polyurethane tube to create a single housing unit. Cages were furnished with suspended PVC tunnels, floor tunnels,

Figure 7: Existing multilevel cages for ferrets can be modified to house large groups of rats.

aspen-wood chew blocks, cardboard tubes, and a large nest box (Figure 7). Staff working with these rats observed them to be calmer, easier to handle, and much less likely to startle when someone entered the room. The rats also displayed species-specific behaviors such as a hopping gait, climbing, jumping, nesting, cooperative hoarding, and extensive foraging.

Another group developed a housing paradigm they call PhenoWorld that doubles as a behavioral monitoring system. This housing unit is approximately 20 inches high with an 11-square-foot floor space and cardboard tunnels. On one side, the arena connects to a box with four running wheels; on another side, it connects to two boxes with food and water that are accessible via an automatic gate. Each running wheel and automatic gate is equipped with antennas that can read implanted RFID tags. A group of six male Wistar Han IGS rats living in PhenoWorld showed clearer circadian patterns of sleep and social interactions compared to groups of two or six rats living in conventional cages (measuring 17 x 10 x 7 in. and 24 x 17 x 8 in., respectively) with cardboard tubes (Castelhano-Carlos et al., 2017).

Concerned for the welfare of rats subjected long term to the "depriving housing standards" of a conventional cage, Roschke et al. (2024) compared various outcome measures in older male Sprague Dawley rats housed in one of two conditions: (1) in pairs in conventional shoebox cages furnished with tunnels, several types of nesting material, gnawing wood, and a plastic shelter; (2) in groups of six in modified, multilevel rabbit cages containing the same items as the shoebox cages, plus a digging box and a running plate. Additionally, half of the animals in each housing condition were placed in a playpen for between 45 minutes and 5 hours, twice a week for 6 months. The playpen was a large (59 x 38 x 20 in.) enclosure consisting of a grid placed over a former feeding cart and containing various resources, including a shallow water bath, a running plate, a running wheel, and a snack board toy (Figure 8). Rats were placed in the playpen as either one six-rat group from a modified rabbit cage or three pairs from the conventional cages. (These three pairs only came together during playtime, and it was noted that, over time, there was a decrease in conflicts between rats from different pairs.) In the playpen, rats from the modified rabbit cage were faster to solve an intelligence toy from which they had to withdraw items and showed less interest in the running wheel and plates than those from the conventional cages. There were no differences in corticosterone levels between the four groups (two cage types, with and without playpen access). Testosterone levels were lowest in the control group (conventionally housed pairs without playpen access), likely because testosterone is influenced by social hierarchy, and these rats had fewer social interactions than rats from the other groups who

either lived or interacted with more individuals. Finally, liver proteins associated with lipid metabolism (cholesterol removal and inflammatory inhibition) were lower in the control group. The authors concluded that because there were no adverse effects on their liver-focused research and that general data variability did not increase, there was no reason to keep rats in conventional cages. While staff initially expressed concerns about the added workload to clean the larger cages, after 2 weeks they reported that larger cages did not take more time and that, in fact, they preferred working with the larger cages because rats living in them seemed more content. Placing rats in playpens while their cages are being cleaned could also be incorporated into standard practice.

Figure 8: In Roschke et al. (2024), older male rats benefited from being housed in modified, multilevel rabbit cages with a digging box and a running plate (left) and from spending time in a playpen that consisted of a grid placed over a former feeding cart containing a shallow water bath and various other resources (right).

While metabolic caging is widely recognized to be a stressful environment, Whittaker et al. (2016) investigated the magnitude and nature of behavioral changes in male Sprague Dawley rats housed within either conventional open-top or metabolic cages. On day 4 of housing within each treatment, rats in metabolic cages spent significantly more time in inactivity (lying, sleeping, crouching) and less time active (moving, climbing, digging) and attentive (rearing, sniffing the cage or air). Notably, rats in the metabolic cages were frequently observed lying in the food hopper, which

could have been an attempt to avoid the grid floor. In the social interaction test, rats in metabolic cages also spent more time inactive and rearing and less time grooming and following, indicating a more anxious affective state. The authors concluded that metabolic caging is likely associated with compromised welfare in rats.

Hydrophobic sand, which is a biodegradable material coated with a nontoxic water-repelling coating, has been proposed as a humane alternative to metabolic caging. Hoffman et al. (2018) assessed urinary and stress markers and urine volume in male Sprague Dawley rats housed on hydrophobic sand or within metabolic cages. Within each treatment, all rats experienced five sessions, each of which lasted either 2, 4, or 6 hours. For the hydrophobic sand treatment, a single 300-gram packet of sand was used in place of bedding in a mouse cage; urine pooled on top of the sand. No differences were found in stress markers (rat weight, number of fecal pellets, urine corticosteroid) or relevant urinary markers (e.g., leukocytes, glucose, bilirubin, pH) between treatments. There was no difference between treatments in the volume of urine collected in the 2-hour session, and because this volume was equivalent to 62% of the volume collected in the 6-hour sessions, two 2-hour sessions would be a refinement over one 6-hour session. Anecdotally, rats in metabolic cages appeared to be less active and showed some difficulty in keeping their feet from getting caught in the grid floor; they also slept in an odd, somewhat upright position with their head tucked into their chest. The authors concluded that hydrophobic sand represents a refinement over metabolic caging because it preserves the quality of urine samples while reducing both the rats' risk of injury and the time they must spend in isolation (since they are subjected to shorter, more precisely timed collection sessions).

Other Rodents

Guinea pigs in laboratories tend to avoid the central area of their cage. A simple and effective way to maximize use of the whole cage is to add a dark-colored cover over the center third of the cage to mimic vegetation that wild guinea pigs would use for cover. When a black foam board measuring 10 x 24 inches was placed over the central portion of their cage lid, male and female albino Hartley guinea pigs spent significantly more time in central sections of the cage compared to when the shader was absent. When the shader was absent, they spent more time around the feeder (Byrd et al., 2016).

Giral et al. (2015) tested the effects of moving male Dunkin-Hartley guinea pigs from static open-top cages to IVCs. Guinea pigs were implanted with RFID tags and subsequently single housed in barren static cages for more than 6 months. Then,

half of the animals were placed into a new static cage and half into a new IVC cage (Tecniplast's Eurostandard Type IV S, 15–20 ACH). There were no differences in heart rate between the two treatments. Guinea pigs in IVCs were significantly less active on the first day, but activity levels equalized thereafter. Over the 4-day observation period, guinea pigs in the IVCs consumed less food, and four of the five animals lost 3–8% of their body weight, compared to only one animal losing 3% body weight in the static cages. The authors concluded that decreased appetite causing weight loss in the IVCs was related to stress, but studies of longer duration and with larger sample sizes are needed to ascertain the importance of this finding. Additionally, it is possible that housing the animals with a companion and a shelter would decrease the impacts of cage ventilation by aiding in thermoregulation.

Housing guinea pigs within larger, multilevel cages can encourage more physical and mental stimulation. However, guinea pigs are not morphologically adapted to climbing, so Begum-Diamond et al. (2022) tested the animals' willingness and ability to use a 4-foot ramp placed at various angles. Using female guinea pigs housed in large enclosures (which contained fleece, hay, wood shavings, and cardboard boxes), they found that, on average, an incline of 13–14 degrees was the steepest angle that guinea pigs were willing to climb. Therefore, when using multilevel housing for guinea

Figure 9: Guinea pigs housed in a floor pen that was set up in a vacant room and furnished with items from within the facility were more willing to interact with humans, were easier to handle, and expressed behaviors indicative of happiness and excitement (King, 2019).

pigs, ramps should not be too steep, to ensure that the animals can access and benefit from all the levels.

In a descriptive study, King (2019) discussed their facility's transition from conventional cages to floor pens for guinea pigs. The conventional cages housed five guinea pigs and were too cramped to accommodate any environmental enrichment. In contrast, the floor pen housed 10 guinea pigs; it was set up in a vacant room and furnished with items from within the facility, including empty boxes and tunnels (Figure 9). Food was provided via hoppers from the conventional cages placed on the floor. The staff noticed immediate changes in the animals' behavior: The guinea pigs were more inquisitive, more willing to interact with humans, and easier to handle. They also frequently engaged in "popcorning," a behavior characterized by sudden and erratic jumping known to be an expression of happiness and excitement in guinea pigs.

Naked mole rats (NMRs) spend their life underground within a maze-like burrow system. These burrows consist largely of shallow foraging tunnels that connect to deeper chambers the size of a football that are used for nesting, food storage, and latrines. Tunnel expansion is guided by the search for food; indeed, rather than foraging above ground, NMRs burrow their way—likely using chemical signals from plants—toward tubers, bulbs, and roots of geophytes (plants with underground storage organs).

There are no commercially available housing systems catering to the needs of mole rats, so NMRs in laboratories must be accommodated through DIY solutions. Due to their propensity to continually expand their burrows in search of food, their housing must be constructed using hard materials that they cannot chew through. Smith and Buffenstein (2021) described using impact-resistant polycarbonate round tubing (2.5-in. outside diameter) to connect several mouse cages to mimic the maze-like structure of a burrow. The minimum number of cages (chambers) is three: one for nesting, one for food storage, and one for toileting. Three chambers are suitable for up to 15 individuals, but up to 25 chambers are recommended for colonies of 150–200. Regardless of colony size, NMRs typically pile on top of each other within a single nesting chamber. However, once a toilet is full in the wild, it is sealed off and a new one is constructed. Thus, the primary purpose of increasing the number of chambers as the colony grows is to keep the colony clean (i.e., allowing NMRs to create more than one toilet chamber). NMRs prefer the toilet to be a blind ending chamber, so a circular design is not recommended. One can also add a "sauna" chamber with a heating pad underneath to mimic a chamber that would be found closer to the surface in the wild. (NMRs are poikilotherms, meaning that they are both endothermic and ectothermic.) If space permits, housing can be constructed using multiple tunnels of various lengths

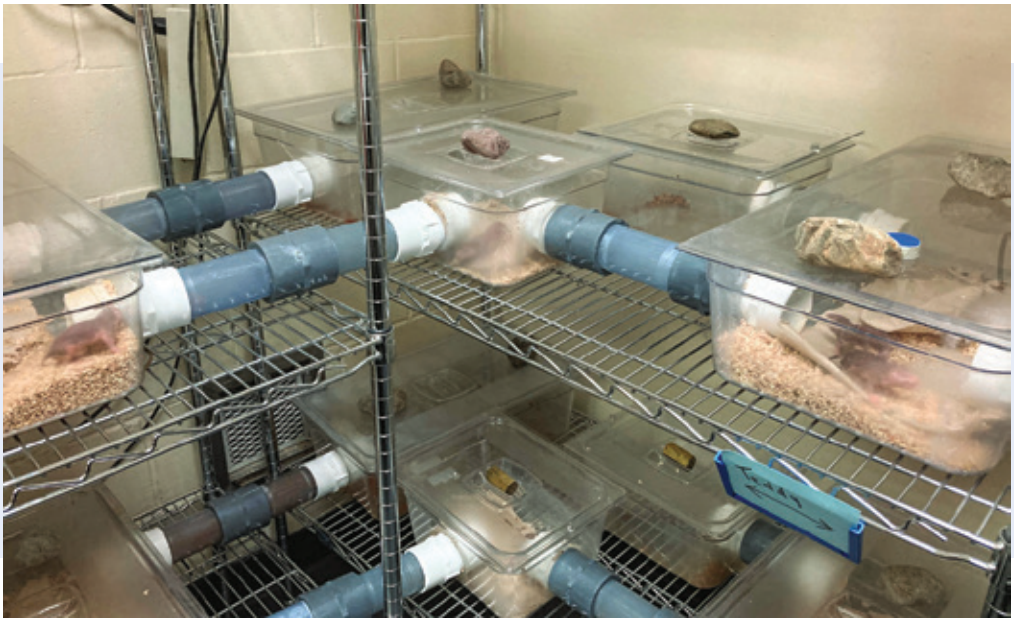

Figure 10: An example of a custom-built housing system for naked mole rats, consisting of 6-inch-deep food pans with lids weighed down with heavy river stones and interconnected via clear 2-inch PVC pipes (Pantophlet et al., 2022).

and chambers over multiple levels. To securely fasten tunnels to chambers and prevent escapes, Smith and Buffenstein (2021) use silicone bands (youth-sized wristbands) on the outside of the round tubing, on both ends, to prevent the tubing from sliding deeper into the mouse cages. Moreover, cages can be prevented from moving on the cage rack using either Velcro or silicone pads underneath them.

Supplemental heat and humidity are often required to maintain levels appropriate for NMRs. To facilitate this, Ragland et al. (2022) described housing over 50 NMRs and up to three colonies inside a temperature-regulated cabinet (Tecniplast Aria Ventilated Cabinet BIO-C36) maintained at approximately 85 °F (29 °C) and 50% humidity. Mazes similar to the ones described above, constructed using mouse cages and impact resistant polycarbonate tunnels, are placed inside the cabinet.

Pantophlet et al. (2022) experienced frequent NMR escapes when using rubber bands to keep tunnels fastened to cages. To remedy this problem, they developed a novel housing system using 6-inch-deep food pans with lids for chambers, and 2-inch clear PVC threaded pipes at schedule 80 rigidity for tunnels (Figure 10). Tunnels are fastened to the pans using either PVC threaded couplings or metal lock nuts; lids are

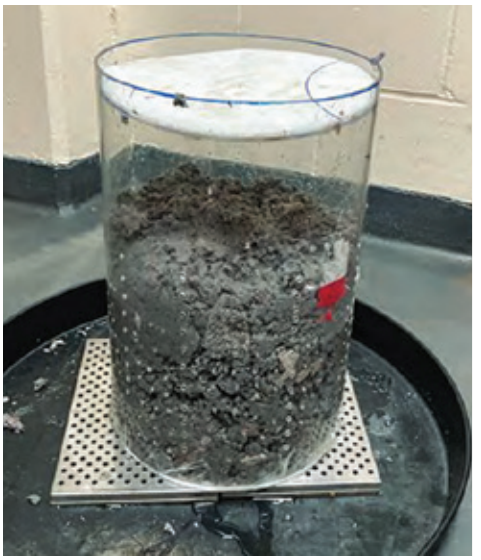

Figure 11 (left): Pantophlet and McClusky (2022) showed that shredded newspaper can be soaked, pulped, compressed, and dried to form a dense paper cylinder suitable for naked mole rats to burrow into. Figure 12 (right): Naked mole rats excavated a spiraling tunnel all the way to the top of the column within the first night (Pantophlet & McClusky, 2022).

kept in place over the pans using either heavy river stones or reptile cage clips. The authors find this new system to be more versatile and easier to maintain compared to their older system. However, they cautioned that the right cleaning agents must be used to prevent some of the components from warping, and that hand threading of some of the elements must be done carefully to ensure proper fit.

NMRs are typically kept in wood shavings, corn husks, corncob, pelleted paper, or soft cellulose bedding (Pantophlet & McClusky, 2022; Ragland et al., 2022; Smith & Buffenstein, 2021). However, these substrates are very different from the hard, solidified lateritic loam soils and pure gypsum found in their natural habitat in East Africa. To better support NMRs' species-typical behaviors, Pantophlet and McClusky (2022) developed a new type of hardened substrate from compressed paper that NMRs can excavate and burrow into. (According to *Encyclopedia Britannica*, 25% of NMRs' muscle mass is contained in the jaw, and nearly 33% of their brain's somatosensory cortex is devoted to their teeth; burrowing is *important* to them.) Briefly, shredded newspaper was soaked in a 0.01% bleach solution for 24–48 hours, and the mixture was then pulped with a paddle until it reached the consistency of porridge. Next, the pulp was poured into a 24-inch-diameter acrylic column with numerous small drainage

holes around its perimeter and its bottom; the pulp was pushed down and compressed over a drain until it formed a dense paper cylinder (Figure 11). The cylinder was left to dry for about a week. The hardened paper column was fitted into another acrylic cylinder with a 2-inch-diameter entry hole drilled near its bottom, so that a tube could be inserted and the column attached to the NMRs' existing colony housing. The top of the column was covered with Plexiglas. The authors introduced NMRs to the column overnight. Within that first night, the animals had excavated a spiraling tunnel all the way to the top of the column (Figure 12). The authors hope to upscale this project to give NMRs complete control over the construction of their burrowing system. In a study supported by an AWI Refinement Research Award, Pantophlet (2024) later confirmed that compressed newspaper columns—whether soaked for 2 hours in reverse osmosis water (untreated) or a solution of bleach, or boiled for 2 hours in reverse osmosis water or a solution of sodium chloride—were safe for NMRs. Specifically, there were no differences in the abundance of bacteria or fungi between the four treatments, nor were there any unique bacteria in the untreated paper compared to an unused sample of corncob bedding. Furthermore, untreated newspaper was found not to contain concerning levels of any nonliving contaminants, such as heavy metals.

Damaraland mole rats (DMRs), who are larger than NMRs, also live within underground tunnel systems but in smaller colonies. Therefore, housing for DMRs should be constructed using rat and mouse cages as chambers and 3-inch-diameter tubing as tunnels. Buffenstein et al. (2024) recommended a minimum of four chambers to house 2–10 individuals. These chambers should be in a U-shaped conformation to create two blind ends, one for use as a toilet and the other as food storage. More chambers should be added with larger colonies to allow DMRs to establish more latrines and more storage chambers for subordinate animals to access food more easily.

Rabbits

Coda et al. (2020) compared the behaviors of single-housed male and female New Zealand white rabbits when they were placed in barren enclosures of different sizes for 120 minutes: a standard cage (25 x 29.5 x 16 in.), a medium cage (28 x 45 x 27 in.), and a large run (65 x 70 x 96 in.). The medium and large enclosures were lined with a rubber mat to prevent rabbits from slipping. Rabbits spent significantly more time exploring in the large run, and less time grooming in the large run and the medium cage, compared to the standard cage. Time spent resting and the frequency of rearing, digging, and grooming did not differ between treatments. The authors concluded that larger cages promoted a broader range of species-typical active behaviors.

Figure 13: In Hedenqvist et al. (2020), rabbits housed within steel-mesh floor pens engaged in more physical activity and experienced better bone healing compared to rabbits in conventional rabbit cages.

Housing rabbits within larger enclosures that allow for more physical activity benefits not only the animals' welfare, but also the validity of data obtained from them. Hedenqvist et al. (2020) compared bone healing in female New Zealand white rabbits used in orthopedic research when the animals were housed according to standard conditions compared to large floor pens. In the standard treatment, rabbits were single housed in a cage with a perforated plastic floor lined with a towel and a combined shelter/resting shelf. In the floor-pen treatment, rabbits were pair housed within steel mesh pens that were seven times larger than the cages; the pens had a plastic floor and were furnished with two shelters, a litter pan, and aspen shavings (Figure 13). All rabbits had free access to straw and hay. Rabbits in the floor pens engaged in higher physical activity and had better bone healing outcomes, leading the authors to conclude that restricted activity in standard cages may have a negative effect on the results of rabbit orthopedic studies.

KEY TAKEAWAYS: HOUSING

- Conventional **rodent** caging was primarily designed for human convenience rather than animal welfare and, as such, is one of the greatest limiting factors to rodents' ability to live a "good life" in the laboratory. Ultimately, the aim should be to move away from small, barren cages to more complex, less restrictive options.
- All **rodents** and **rabbits** would benefit from greater space allocation, *assuming increased space is accompanied by increased environmental complexity*. Greater floor area—without more resources, shelters, enrichments, and furnishings—will not, on its own, result in a marked improvement to welfare.
- **Mice** are commonly housed in IVCs, which are drafty and can induce cold stress in mice. Draft-related cold stress can be reduced by providing sufficient nesting material, a heated floor, and/or air delivery at the cage-lid level rather than at the animal level. Mice also benefit from distinct areas within their enclosure that allow them to self-designate sleeping, eating, and latrine areas.
- Welfare-friendly commercial laboratory cages are not readily available for **rat** housing. Researchers can fashion their own welfare-friendly housing setups using pet rat cages or repurposed ferret or rabbit laboratory cages. Cages should be furnished with items that allow for burrowing, climbing, and upright standing.
- Where possible, metabolic caging should be avoided for **rat** housing; hydrophobic sand is a more humane alternative.
- A dark "shader" can be placed over the middle third of a conventional **guinea pig** cage to encourage use of the cage's central area. Floor pens are appropriate for guinea pigs; if the setup provides multiple levels, the incline of access ramps should not exceed 13–14 degrees to facilitate use.
- There are no commercially available housing systems to accommodate the needs of **mole rats**, who evolved to spend their life within underground, maze-like burrow systems. To keep these animals within laboratories, researchers must use rodent cages and round tubing to custom-build housing systems simulating a network of tunnels and chambers. Mole rats can be given the opportunity to excavate tunnels via custom-made compressed paper columns.
- Floor pens for **rabbits** promote physical activity and are likely associated with better welfare and better study outcomes.

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Social Housing

What Is It, And Why Does It Matter?

Most species of rodents and rabbits are gregarious, which means that conspecifics have a proclivity to interact and cooperate with each other. When these species are in captivity, social companionship is widely regarded as the single most important feature of their environment, because it provides continuous and dynamic stimulation (Sauer, 2004).

Keeping social animals in isolation has numerous dramatic and long-term negative effects on their welfare, including on their development, physical and mental health, and behavior. When animals are housed alone in laboratories, this is often out of the belief that single housing simply removes the companion as an experimental variable. However, this belief is misguided. One cannot "remove" an animal's environment; one can only make an environment better or worse. Single housing certainly removes the influence of the companion, but what's left in its absence—social isolation—is itself a significant experimental variable with many negative downstream effects on various biological and psychological parameters, data quality, and translational validity (Pinnell et al., 2016). For example, single housing of mice used as a model of ischemic stroke decreases survival rate, exacerbates the damage caused by the stroke, and alters neuroinflammatory responses (Karelina et al., 2009; Verma et al., 2014); single housing of transgenic mice used as a model of Alzheimer's disease worsens cognitive deficits and the number of plaques in the hippocampus, and these effects cannot be alleviated by the provision of a running wheel or other environmental enrichment (Peterman et al., 2020); and social isolation of mice and rats used as models of epilepsy leads to a substantially greater number of seizures, higher anxiety, and lower cognitive performance (Manouze et al., 2019).

Laboratory studies have shown that mice, rats, and rabbits actively seek out social partners (Figure 1). Both male and female mice, for example, worked harder for contact with a familiar mouse than for food (Ramsey et al., 2023) and, in conditioned place-preference (CPP) tests, preferred compartments where they had resided with other mice rather than ones where they had resided alone (Panksepp & Lahvis, 2007); female hooded rats worked harder to access a cage with familiar rats than they did to access a larger cage or one containing various enrichment items (Patterson-Kane et al., 2002); in CPP tests, both male and female adolescent Sprague Dawley rats preferred locations where they had interacted with other rats (Douglas et al., 2004); and female New Zealand white rabbits worked almost as much for contact with an unfamiliar rabbit through a wire mesh partition as they worked for food (Seaman et al., 2008). In mice,

Figure 1: Most species of rodents and rabbits are gregarious and seek out social partners. Left: Two rabbits retired from a laboratory enjoying each other's company at a private shelter; Right: Five rats in semi-naturalistic laboratory housing sharing a hammock.

motivation for social contact varies between strains, and some evidence suggests preference may be stronger for siblings than non-siblings (Harda et al., 2022). Syrian hamsters are widely viewed as solitary, but this may not be true—especially for males. Like mice and rats, male Syrian hamsters in CPP tests also preferred locations where they had interacted with another hamster, and these social interactions stimulated the reward system in the brain (Gil et al., 2013; Song et al., 2016).

An important benefit of living with conspecifics is that it allows animals to better cope with stress (such as surgery or disease), a phenomenon known as social buffering. The presence of a companion offers distraction, comfort, and the chance to experience positive "emotional contagion"—whereby one's emotional state is influenced by and ultimately reflects that of proximate individuals (Denommé & Mason, 2022). Negative emotional contagion can also occur. However, while emotional contagion of fear may induce avoidance of the fearful companion, emotional contagion of pain can actually be beneficial, as it promotes physical closeness and caring and helping behaviors (Du et al., 2024). At a molecular level, positive sensory cues (e.g., tactile, visual, olfactory, and auditory) from conspecifics lead to the release of oxytocin—a neuropeptide whose cascade of effects includes

increased social bonding and decreased stress (Kikusui et al., 2006), often resulting in better recovery from surgical procedures or other trauma.

Even for animals who are socially housed, social isolation after surgery is a common strategy to protect wound sites and minimize interference with exteriorized devices, such as telemetry equipment. However, social isolation slows postsurgical recovery and may actually reduce the validity of data collected from preclinical animal models for human applications, since humans typically have access to social companionship during recovery (Jirkof, 2015). Hawkins (2014) makes the point that, following surgery, physical harm or loss of data points *may* occur in social housing, while the physiological and psychological harm of single housing *will* occur. Precautions can be taken to decrease the likelihood of physical damage, such as by refining suturing techniques to use intradermal sutures that are less conspicuous. If animals need to wear exteriorized telemetry devices or protective jackets, those could be introduced to the animals ahead of time, so they are habituated to them by the time they are needed.

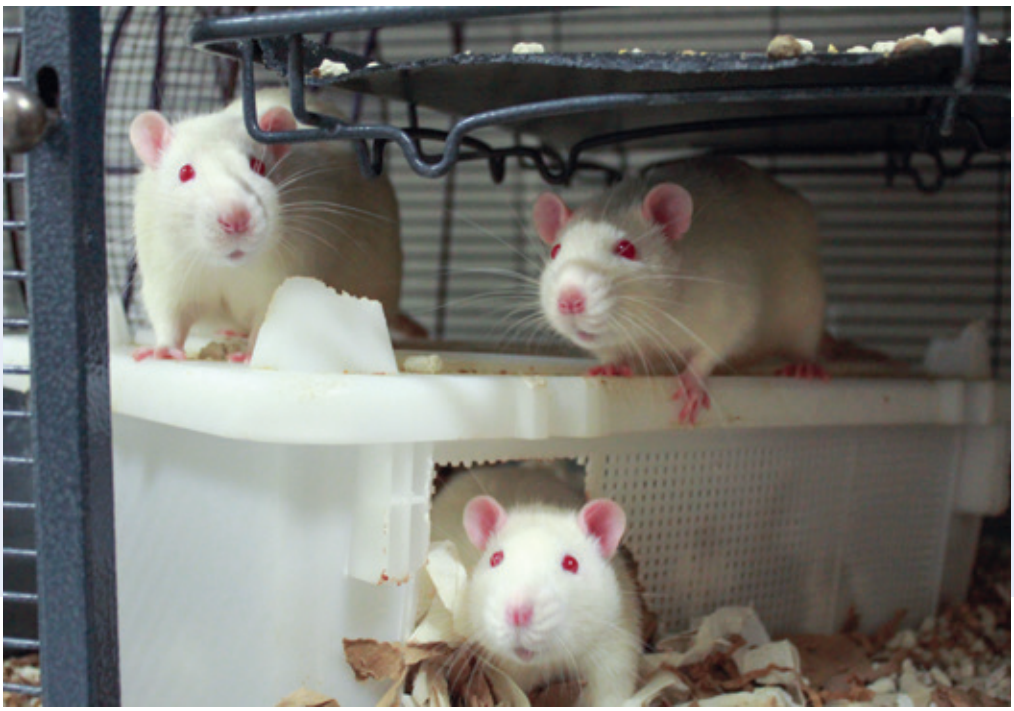

Figure 2: Successful social housing of rodents and rabbits may require creative management strategies and a willingness to alter the way in which these species are housed—both of which are well worth the effort and investment.

While most rodents and rabbits benefit greatly from social companionship, it is important for cagemates to get along, because the *quality* of the social relationship affects current and long-term welfare and development (Gudsnuk & Champagne, 2012). Aggression among cagemates is an important welfare concern, especially for animals who are recipients of recurring agonism. A likely underlying cause of aggression in laboratories are the restrictive and unnatural conditions under which rodents and rabbits are typically held (DiVincenti & Rehrig, 2017; Weber et al., 2017). Conventional laboratory environments deprive caged animals of social control and prevent them from engaging in species-typical social interactions. Specifically, animals cannot choose their social companions, and they cannot engage in effective strategies to de-escalate an aggressive event. Short of fundamental changes to how rodents and rabbits are housed, various strategies (discussed below) can be implemented to minimize aggressive encounters in laboratory housing.

The positive effects of social housing and negative effects of single housing are well studied, and to describe them all would require an entire book of its own. Instead, the focus of this chapter is on strategies to achieve harmonious social groups, as well as strategies to mitigate the negative welfare effects to animals who may be single housed because of the challenges specific to certain experimental manipulations. In some situations, successful social housing of rodents and rabbits may require creative management strategies or willingness to alter the way in which we house these species—all of which are well worth the effort and investment (Figure 2).

Summaries of Current Refinement Research

Mice

GENERAL STRATEGIES

Studies of wild house mice have revealed a variety of social structures. In some populations, individual males defend a territory from other males, while females freely move between them; in others, several males share the same territory but maintain a stable dominance hierarchy; in still others, the majority of males do not have a

territory, but live relatively peacefully between territories defended by a few dominant individuals (Weber et al., 2017). At research institutions, aggression among males is "one of the most serious welfare concerns in laboratory mouse husbandry" (Weber et al., 2017); as such, the bulk of research on social housing for mice has focused on strategies to minimize aggression. Aggression can lead to pain and injury; indeed, fight wounds are among the most commonly reported health issues in mice (Marx et al., 2013), often resulting in separation or euthanasia.

Two groups of scientists came to the conclusion that aggression in group-housed mice may never be eliminated unless we fundamentally change the way in which we house these animals (Kappel et al., 2017; Weber et al., 2017). Shoebox cages largely strip mice of control over their physical and social environments; these small cages prevent them from engaging in many highly motivated behaviors, such as burrowing, foraging, dispersal, and defense of territories. A chronic inability to engage in these activities results in stress, frustration, boredom, and disturbed social behavior, ultimately contributing to aggressive behavior. Both groups of scientists nonetheless provided insights for addressing aggression in shoebox cages, since this housing system is widely used in today's laboratories. Kappel et al. provided a thoughtful discussion concerning whether it is better for male mice to be housed singly or in groups, given that there are negative impacts of both isolation and aggression. For example, group-housing allows for the expression of important social behaviors, but persistent fighting causes wounding and chronic stress, especially for those on the receiving end of the aggression.

From an animal welfare standpoint, we generally recommend group housing male mice, but there are many context-specific caveats to this recommendation, such as age, strain, familiarity between individuals, and husbandry procedures. Weber et al. reviewed the scientific literature on social housing of mice and determined that strategies to minimize aggression generally focus on three topics: manipulation of group size and composition, husbandry, and resource distribution (e.g., environmental enrichment). Findings from these approaches suggest that, at minimum, mice should be kept in stable social groups of up to three males at weaning (keeping littermates together when possible), providing sufficient nesting material, transferring used nesting material (but not bedding) at cage changing, and minimizing disruptions, especially during the light phase when mice are sleeping.

The personal experiences and observations of animal technicians, veterinarians, and researchers in Sweden appear to corroborate the minimum recommendations outlined by the authors above. In one article, researchers conducting long-term

toxicity studies outlined a variety of strategies adopted by their team to minimize aggression in group-housed male CD1 mice (Annas et al., 2013). The strategies include forming social groups before mice reach sexual maturity, transferring used nesting material at cage changing, and keeping external variables constant (e.g., no changes to noise, light intensity, or the cages' position in the room). The key is to maintain stable group dynamics/social hierarchies. The removal of individuals for breeding—but not for brief biological sampling or injections—may result in increased aggression upon reintroduction to the group. Moreover, males should be handled before females, with gloves changed between sexes, to avoid aggression between males due to the introduction of female scent marks. Finally, one way to identify potential aggression early on is to look for a disordered nest: A nest that is flattened or spread out at the start of the light phase may indicate that fighting occurred during the dark phase. Similarly, Zidar et al. (2019) asked mouse users working at universities, pharmaceutical companies, and governmental agencies in Sweden about their experiences with mouse social housing. Users shared that aggression and resulting injuries were the most common problems associated with group housing, and that C57BL/6 mice were perceived as the most challenging strain to house in groups. They shared a perception that aggression was minimized when mice were housed in stable groups (avoiding regrouping), when group formation occurred before mice reached sexual maturity, and when nesting material or other enrichment items were transferred at cage changing.

Two studies quantified the prevalence of mouse aggression and sought to correlate its occurrence with other factors. The first study was a data crowdsourcing project that included 44 facilities (accounting for 45,400 cages) predominantly located in the United Kingdom (Lidster et al., 2019). Technicians were asked to record aggression-related injuries during daily routine cage checks for 4 consecutive weeks. Injuries were seen in about 3% of cages, with most aggression-related incidents occurring in individually ventilated cages (IVCs). Transferring used nesting material (but not bedding) at cage change and keeping mice with littermates helped to minimize aggression-related injuries. Strains showing the highest prevalence of aggression were CD1, CBA, and C3H, and those showing the lowest were C57BL/6, BALB/c, and 129S. (Interestingly, as noted above, users in Sweden reported that C57BL/6 were the most challenging strain to group house; in the United Kingdom, this strain was found to be one of the easiest. The variability in user experiences with this strain is prevalent throughout the world. Most likely, differences in stock (e.g., which vendor the strain comes from) and in local housing and husbandry standards play a role in this variability.) The highest rates of aggression-related injuries occurred in cages with three mice; the authors acknowledged that this finding was surprising, given that previous research in the 1980s and 2000s had suggested that housing mice in groups of three to five could

Figure 3: Keeping mice of different strains and coat colors together is a simple and noninvasive way to identify individuals, provided that incompatible phenotypes are not mixed.

minimize aggression. (And, as noted above, Weber et al. (2017) recommended groups of *up to* three mice.) The authors speculated that, due to their use of different approaches and strains, their findings may not be directly comparable to those of previous studies.

The second study was an epidemiological investigation of 2,679 cages at Stanford University over the course of 12 months (Theil et al., 2020). Fighting was almost exclusively observed in males. While aggression was observed in approximately 14% of cages, visible injuries were detected in only 14% of those 14% (i.e., about 2% of total cages), suggesting that brief cage-side visual checks are likely inadequate for gauging the true prevalence of aggression-related injuries. Housing parameters were the largest contributor to aggression between male mice; specifically, IVCs with corncob bedding had significantly higher aggression rates than static cages with woodchip bedding. However, no IVCs contained woodchip bedding and few static cages had corncob bedding, so it is not possible to separate the effects of cage type versus bedding type. Other factors, such as type of nesting material, presence of a shelter, ear notching, the number of mice in the cage, and experimentally induced morbidity all failed to predict fighting. Because of the large impact of housing parameters on aggression and wounding, the authors highlighted that animal welfare costs should be considered more seriously when investing in new housing systems.

When group housing mice, keeping those of different strains and coat colors together is a simple and noninvasive way to identify individuals (Figure 3). Walker et al. (2016)

demonstrated that female C57BL/6, DBA/2, and BALB/c mice can be housed together in mixed-strain trios without compromising mouse welfare (e.g., no changes in stereotypic behavior, aggression, or corticosterone metabolites) or increasing the variance in experimental outcomes. Housing mice in this manner also allows several strains to be tested at once; it is an example of a split-plot design that allows fewer animals to be used while achieving the same statistical power. However, another study suggested that mixed-strain housing may result in higher levels of social stress for some mice. Kulesskaya et al. (2014) compared the effects of single-strain versus mixed-strain housing of female C57BL/6 and DBA/2 mice. These strains tend to exhibit different behavioral phenotypes, which may impact how they interact with each other. The study found that both strains, when housed together, exhibited higher physiological signs of stress (e.g., elevated serum corticosterone) compared to when each strain was housed separately. Additionally, DBA/2 mice were behaviorally unaffected by mixed-strain housing, but C57BL/6 mice showed altered learning and social behavior, and increased anxiety and anhedonia. Therefore, some strains may be more compatible than others in a mixed-strain housing scenario.

A number of experiments investigated strategies that contribute to successful pair or group formation. Jirkof et al. (2020) found that male CD1 mice housed in groups of three had lower wounding scores than pair-housed mice, and that single-housed mice showed signs of impaired welfare (lower body weight despite higher food consumption; increased sucrose consumption). Azkona and Caballero (2019) achieved success by incorporating a newly weaned male mouse (~3 weeks old) into an existing cage of males up to 1 week older (~4 weeks old); the authors tested this with CD1, SCID/Beige, C57BL/6, NOD SCID, and various genetically modified strains. Grífols et al. (2020) had similar success incorporating a newly weaned male mouse into an existing cage of juveniles up to 2 weeks older (~5 weeks old) in CD1, SCID/Beige, and C57BL/6 mice. However, in C57BL/6 mice, incorporating a weanling into a cage of older mice (~5 to 7 weeks old) resulted in aggression, sometimes causing death. Reading et al. (2020) found that hanging a mirror in the cage of single-housed mice a few days before pairing with another mouse can increase pairing success. The rationale is that it may help mice become accustomed to seeing another mouse in their cage. The disc-shaped mirror is hung centrally from the lid of each cage; additionally, small amounts of bedding and nesting material are exchanged between the two cages to familiarize the mice with one another's scent over the course of 2 to 3 days. The authors had a 78% pairing success rate using this method, compared to 16% if no mirror was used.

While mirrors may help to improve pairing success, they should not be used as a stand-in for social housing. Fuss et al. (2013) placed a mirror covering the entire rear

wall of the cage of single-housed male and female C57BL/6 mice to simulate social housing. The mirror had no discernable effect on anxiety or depressive behavior, visual memory, or neural proteins that play a role in cognitive function and mood. The mice also showed no spatial preference for a compartment containing the mirror. Caretakers, therefore, should not assume that mirrors or visual cues of other mice can take the place of actual social interactions.

When male mice are kept long-term for nonexperimental purposes, such as for training programs, castration may be an option. Groups of 10 male Swiss mice were either surgically castrated after puberty or left intact (Vaughan et al., 2014). Intact mice had significantly more aggressive events occur at all points in time over a 3-month observation period and had notably worse coat conditions by the end of the study. This approach may be a refinement in circumstances where male mice are maintained in long-term group-housing scenarios, but the cost of putting the animals through an invasive procedure must be considered.

Adding partial cage dividers is emerging as another strategy to reduce aggression in group-housed males. In one study, groups of three male BALB/c mice were housed

Figure 4: Housing male mice with partial cage dividers can help reduce aggression and wounding and enhance the stability of social hierarchies.

with or without an opaque, white divider made of corrugated plastic inserted through the wire lid (Tallent et al., 2018). The divider separated one half of the cage into three partially separated areas, so the cage was made up of one larger common area and three small rectangular "burrows." Mice were able to thwart an aggressive encounter by escaping into one of the burrow areas; the addition of these cage dividers led to significantly fewer aggressive interactions. A different experiment found that, although partial cage dividers may not reduce aggression, they may contribute nonetheless to more stable social ranks, which helps to reduce phenotypic variability between individual mice (Streiff et al., 2024). Male BALB/c and Swiss mice were housed in groups of three or five individuals, with or without two red, transparent cage dividers (Figure 4). In BALB/c mice, cage dividers decreased the number of aggressive events, while in Swiss mice, dividers increased them. However, in both strains, rates of wounding and hormone levels (testosterone, which modulates aggressive behavior, and fecal and hair corticosterone, which is a stress hormone) were similar between the two treatments. Interestingly, dominance hierarchies differed between cages with and those without dividers: In cages with dividers, dominant mice were less despotic, social ranks remained more stable throughout the study period, and there were no hormonal differences between dominant and subordinate mice. (In cages without dividers, dominant mice had lower testosterone and higher hair corticosterone than subordinate mice.) Taken as a whole, these results suggest that partial cage dividers are beneficial for male mice.

Other studies have examined the effects of using full cage dividers—that is, splitting cages in half using transparent, perforated partitions—to physically separate mice but allow them to see, smell, and hear each other. In Buckinx et al. (2021), C57BL/6JRj male pairs divided by a partition did not differ from those who were either single housed or housed in pairs without a partition on measures of anxiety (open field test, elevated plus maze test, hypothalamic-adrenal-pituitary (HPA) axis activity, organ weights) or working memory (Y-maze spontaneous alternation test). Another study found similar results: Pairs of C57BL/6JRj male mice separated by a transparent, perforated divider that split the cage in half did not differ from those who were single- or group-housed without cage dividers on measures of anxiety (free exploration, hair and fecal corticosterone), social behavior (social interaction test), burrowing performance, or ease of handling (Hohlbaum et al., 2022). However, group-housed mice built higher quality nests and had higher body weights, while pairs separated by a divider tended to build their nests in close proximity to each other along the dividing wall, which may indicate a preference for warmth or resting close to a social partner. Thus, transparent, perforated cage dividers do not seem to affect anxiety and a few other measures compared to either single or pair housing;

however, they may offer some opportunities for warmth (though not as much as pair housing). On the flip side, using a cage divider reduces the space—which is already very restrictive—by half for each mouse.

Husbandry and management practices can also help prevent or mitigate aggression. Blankenberger et al. (2018) tested the effect of separating various strains of male mice with bite wounds into smaller groups. They assessed whether splitting cages of four or five males into groups of two or three mice would reduce aggression. The newly separated cages containing the unwounded mouse from the original cage showed slower improvement in wound scores, indicating that this mouse was likely the main aggressor. Nonetheless, fight wounds improved in both separated groups, indicating that this can be an effective management strategy to reduce fighting and to keep male mice from larger groups with at least one social partner. Gaskill et al. (2017) found that weaning at a later age (4 weeks, compared to 2 or 3 weeks) in C57BL/6 males resulted in higher levels of injurious aggression. Contrary to what they predicted, aggression-related injuries were not higher in groups mixed after weaning compared to those kept stable after weaning.

Altering the type and amount of resources within cages is another strategy that can help address aggression. Sherrill and Kavanagh (2019) found that when C57BL/6 male mice were housed in groups of five with a plastic hut, a wooden gnawing stick, and paper nesting material that was provided in pouches, 14 of 60 mice required separation. Four mice were euthanized due to fight wounds. Mice demonstrated less severe aggression when researchers made the following changes: groups of four instead of five, no plastic hut, nesting material provided loosely rather than in pouches, dirty nesting material transferred at cage change, and all cage changes performed by one dedicated female researcher. Mice still showed aggression after cage changing, but there were fewer fights overall and no separations were deemed necessary under these conditions. Menke et al. (2018) found that the addition of several types of enrichment significantly decreased fighting in male C3H/HeJ housed in groups of five. Specifically, fighting was reduced when mice were provided with a hut, 1 cup of crinkle paper, a cotton Nestlet, and a cardboard tube, compared to the standard of a hut and either 1/8 cup of crinkle paper or a cotton Nestlet. Gaskill et al. (2017) found no difference in wound scores between male C57BL/6 mice housed in groups of five within otherwise barren cages containing either a hut, 50 grams of irradiated sunflower seeds dispersed in their bedding, or 8–10 grams of crinkle paper. In another study, trios of male C57BL/6NCrl mice were housed either with or without a cross-shaped divider object that was either transparent or opaque and roughly the size of a plastic shelter (Mertens et al.,

2020). The authors found that the opacity of the dividers made a significant difference: Opaque objects were associated with more stress-related behavioral and physiological parameters compared to transparent objects or no object. Moreover, there was little aggression prior to 8 weeks of age, but after this point, mice with opaque objects behaved more aggressively after cage changing compared to mice with transparent objects or no object. The opaque objects likely led to increased territorial behavior without achieving the goal of providing viable opportunities for the mice to escape each other.

Wang et al. (2024) took an unusual approach to social housing: Male C57BL/6J mice were housed in groups of three immediately after weaning, but the composition of each trio was changed twice a week for 7 weeks. (This model is called "companion rotation" and is used to simulate the high levels of social interaction common in human adolescents.) The authors found that, compared to control animals who remained in stable groups of three, those in the rotation treatment had several indicators of lower anxiety in the open field test, the elevated plus maze test, and the light-dark box. Aggression was not assessed, but would be a clear concern when mixing unfamiliar adults.

CHALLENGES SPECIFIC TO CERTAIN EXPERIMENTAL MANIPULATIONS

Tirado-Muñiz et al. (2023) investigated the effects of single versus pair housing on surgical site healing and general well-being in female C57BL/6 mice after surgery. They found that pair-housed mice had better quality nests after surgery and better scores both before and after surgery on the time-to-integrate-to-nest test ("TINT," a cage-side assessment of the time it takes to incorporate new material into a nest that can be used as an indicator of mouse welfare; Rock et al., 2014). Other measures (body condition, grimace, and wound scores, as well as number of missing wound clips) didn't differ between treatments. These results suggest that pair housing is not deleterious to mice after surgery, and, indeed, offers some benefits.

Moreover, one recent study found that pair-housed mice used in wound healing studies had different wound dynamics than single-housed mice, and that paired mice produced more consistent and reliable results (Yampolsky et al., 2025). Specifically, single-housed mice had overgrown scabs that concealed wound closure dynamics beneath them, which affected measurement consistency. When mice were paired, partners reliably licked and removed scabbed material, which resulted in moderate scabbing and more consistent wound measurements.

Rats

GENERAL STRATEGIES

Wild Norway rats live in large colonies subdivided into smaller populations that share one burrow. Small groups typically consist of a male and 5–10 females (Calhoun, 1963). While males do not typically live in close-knit groups with each other, injurious aggression among socially housed male rats is of relatively less concern than it is for male mice, and rats tend to live harmoniously when they are kept in stable groups.

Perforated cage dividers that allow for visual, auditory, and olfactory contact are useful during introductions of unfamiliar rats but are likely unsuitable as a substitute for social housing. Stryjek and Modlinska (2022) evaluated the use of an autoclavable mesh partition to expose wild male Norway rats to one another for 2–4 days before they were physically housed together. Compared to rats paired without any pre-exposure, rats pre-exposed via the mesh partitions displayed less boxing, pinning, and lateral threat displays upon full introduction to their social partner. The study found no differences in affiliative behavior between treatments, and no differences in behavior between rats paired on clean versus soiled bedding.

Longer-term, full physical contact appears to be necessary for rats to derive the full benefits of social companionship. Nakayasu and Kato (2011) used a wire mesh partition to investigate whether full physical contact is necessary to achieve the positive effects of social buffering for rats who experienced a social stressor. Male Wistar rats who experienced social defeat in a resident-intruder test were subsequently housed in one of three conditions: singly, with a familiar social partner separated by a mesh partition, or with a familiar social partner and full physical contact. When tested in the elevated plus maze test 2 weeks later, rats with full physical contact had significantly lower anxiety compared to the other groups. The authors concluded that physical contact with a social partner is necessary for social buffering to occur. However, because anxiety testing occurred a full 2 weeks after social defeat, it is possible that reduced anxiety is more reflective of the general benefits of living in physical contact with a familiar partner, rather than social buffering following a social stressor.

Peartree et al. (2012) found that, in CPP tests, adolescent male Sprague Dawley rats developed the strongest preference for locations where they could interact with another rat, followed by locations where another rat was behind a mesh partition, followed by locations where they could interact with a ball. Marquardt et al. (2023)

also assessed the importance of full physical contact between adolescent rats. Male and female Sprague Dawley rats were housed for several weeks either alone, in pairs, or in pairs separated by a Plexiglas divider with holes, thereby allowing olfactory, visual, auditory, and limited tactile communication, but disallowing more complex physical interactions, such as play. Male rats separated by a partition as juveniles displayed decreased sexual behavior, increased aggression, and heightened preference for social contact as adults. Both males and females had impaired performance in a prosocial behavior test, indicating that a lack of physical contact generally, or play as juveniles more specifically, may decrease motivation or ability to complete an empathy-driven task. (It must be noted that neither study using perforated partitions controlled for for the fact that the partition also reduced by half the space available to each rat. Therefore, these studies are testing the combined effect of reduced physical contact and reduced space.) Another study—this one using male Lister hooded rats—found that, although rats who were isolated only during adulthood displayed the same behavioral and molecular phenotypes as rats who were isolated from weaning onwards, some of the deficits in neuroplasticity and behavior induced by the latter group's isolation during adolescence were not attenuated by a later period of social housing (Begni et al., 2020). These findings corroborate the finding that rats may be particularly sensitive to isolation during the juvenile period.

CHALLENGES SPECIFIC TO CERTAIN EXPERIMENTAL MANIPULATIONS

To mitigate the risks associated with social housing of rats with cranial implants, Pinnell et al. (2016) developed a 3D-printed ring (0.5 in. inner diameter and 0.2 in. high) that fits around the headstage and attaches to the skull. A removable, stainless steel sewing thimble that covers the headstage is then attached to the ring to act as a protective headcap. Pair-housed female Sprague Dawley rats wearing the protective headcap experienced no damage or complications for 3 weeks after implantation. They were able to engage in a range of normal behaviors, such as grooming and foraging.

After surgery, cagemates sometimes chew on each other’s metal wound clips, which causes them to fall out. To overcome this issue, Thomson and Mungall (2019) recommend suturing skin closed using subcuticular stitching finished with an Aberdeen knot. This method takes approximately 10–15 minutes longer per rat but eliminates the issue of wound clips coming out.

According to Skinner et al. (2019), studies using telemetry devices are routinely granted exemptions from social housing requirements. To assess the prevalence

of social housing for rats used in telemetry studies, the authors surveyed 24 pharmaceutical and contract research organizations in Europe and outside of Europe (this included the United States, Canada, and the United Kingdom). They found that in Europe, all rats used in telemetry studies were socially housed on nonrecording days, and 79% were socially housed on recording days. Outside of Europe, only 50% were socially housed on nonrecording days, and 14% on recording days. The percentages from Europe clearly show that social housing of rats in telemetry studies is feasible, and efforts must be made to ensure social housing for all rats.

To provide a working example of social housing in telemetered rats, Skinner et al. (2019) described a study in which they paired male Han Wistar rats, one of whom was implanted with a C5-PXT transmitter while the other was not instrumented. Rats were housed either in standard IVCs or in double-decker IVCs, which allowed upright standing due to increased vertical space. Continuous physiological recordings were successful in both types of cages, with minimal signal drop-out: 0.3% and 0.1% in standard and double-decker cages, respectively. There were no differences in the detection of drug-related cardiovascular responses when comparing data between the two cage types. Additionally, a preliminary power analysis using pooled data found that there were no differences in the ability to detect cardiovascular changes in pair-housed rats compared to single-housed rats.

Social housing of rats wearing external telemetry devices is also possible. Typically, external electrocardiogram (ECG) electrodes that measure cardiovascular parameters are covered and held in place by a jacket. The DECRO jacket, however, is itself embedded with thoracic and abdominal sensors and an accelerometer, which measure respiratory inductive plethysmography (RIP, a measure of respiratory volume) and general activity, respectively. Fares et al. (2022) tested whether the DECRO jacket used in combination with external ECG electrodes could be used successfully in pair-housed male Han Wistar rats to record cardiovascular and respiratory parameters simultaneously. They found that over the course of three 22-hour recording sessions and two shorter sessions, the amount of ECG and RIP signal acquired generally ranged from 85% to 100%, except for a few instances during the dark phase when rats were especially active, which may have led to displacement of the wires and subsequent gnawing. Rats showed no signs of discomfort or impaired activity when wearing the DECRO jackets, which were made of soft, stretchy material. To habituate rats to these jackets, a piece of fabric similar to that of the jacket was first placed in the rats' cages for a full day. Then, rats were fitted with the jackets for 3 to 5 hours on one day, and for up to 8 hours the following day, before wearing

the jacket for 22 hours. The authors concluded that this noninvasive solution can be used successfully in freely moving, pair-housed rats, paving the way for more humane nonclinical safety assessments.

Rat activity and core temperature are commonly measured in research and regulatory testing, but obtaining these measurements requires single housing when using conventional methods. To remedy this issue, Redfern et al. (2017) developed technology that can track ambulatory activity and subcutaneous temperature of socially housed rats using an automated home-cage recording system dubbed "Rodent Big Brother." Trios of male Han Wistar rats were implanted with subcutaneous radiofrequency transponders that could be detected by antennas positioned under the home cage. Cages were monitored via high-definition infrared cameras. This allowed for continuous monitoring of individuals' movement and temperature without disturbing the animals. The system was able to detect changes in activity related to procedures such as oral gavage and cage changing. Implanting the transponder was easiest in the interscapular region (i.e., the back of the neck) due to the amount of loose skin in that area, but the performance of the system in that position was the worst. Ventral midline placement of the transponder had the best performance but requires a more invasive surgery with more difficult implantation.

Data from rats who are *tested* with their social companion rather than by themselves may also be more scientifically valid. Indeed, de França Malheiros et al. (2021) found that male Wistar rats performed better on a memory test that required spontaneous exploration of objects when they were tested with one of their cagemates. The presence of a social companion during testing generally led to rats exhibiting more exploratory behavior and less anxious self-grooming in the testing environment; there were no differences in object exploration between dominant and subordinate animals, and no instances of agonistic behavior. The authors suggest that in tests of memory, the performance of individually tested rats is impaired by neophobia or anxiety, which can be tempered by a social companion.

Other Rodents

Chinchillas are gregarious animals, but they can be territorial. In the wild, they live in large herds within burrows. As such, chinchillas in laboratories should be housed in groups or pairs, but groups should be formed as soon as possible, and regrouping should be avoided (LaFleur & Williams-Fritze, 2020). Participants in AWI's online discussion forum (LAREF) shared that chinchillas do well when housed in the groups

in which they arrive, even if not all individuals are of the same age. They also indicated that chinchillas like to rough-and-tumble, and that it is very entertaining to watch them play (Reinhardt, 2020).

Degus are highly social animals who tend to forage and nest with unrelated individuals in the wild. In a social preference test, Insel et al. (2020) found that female degus preferred to be near a conspecific, with no clear preference for familiar versus unfamiliar partners. Most degus in this study exhibited no aggression, but some were very aggressive toward unfamiliar animals. Thus, degus are highly social animals and females should be housed in stable same-sex groups; more research is needed on the social housing of males.

Guinea pig pups produce distress whistles when isolated from conspecifics. Tokumaru et al. (2015) found that provision of a companion (mother, father, sibling, or unfamiliar male guinea pig) significantly reduced the distress whistles of isolated pups, with adult companions having more of an impact than siblings. This demonstrates that the presence of a social companion is beneficial for reducing pup distress, regardless of familiarity, because guinea pigs likely feel safest in groups.

Vole species are generally gregarious, but **bank voles** become solitary during the breeding season (Bujalska & Saitoh, 2000, as cited in Kapusta et al., 2022). In **prairie voles**, the presence of a bonded social partner can reduce the impact of a stressful event. Same-sex pairs of male and female prairie voles were subjected to 1 hour of restraint* either alone or with their social partner freely moving in the cage (Donovan et al., 2018). Voles restrained alone demonstrated more anxiety in the elevated plus maze test and significantly lower levels of oxytocin receptor binding (which plays a role in stress coping) in specific regions of the brain, suggesting that social partners can reduce the negative effects of stress through social buffering. Prairie voles may benefit from social support during stressful laboratory procedures; whether their social partners are negatively affected by this exposure, however, is unclear.

Hamsters are territorial and are generally thought to be asocial or solitary animals, but there is evidence that they, too, may benefit from social housing. CPP testing has shown that male **Syrian hamsters** prefer places where they had interacted with another hamster and that these interactions stimulate the reward systems

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** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

in the brain (Gil et al., 2013; Song et al., 2016). Building on this, Ross et al. (2017) investigated the effects of social versus single housing on male and female Syrian hamster physiology. They found that both sexes tolerated stable social groups of five individuals for 4 weeks, with no signs of wounding or tissue damage, and no increase in serum or fecal cortisol. Single-housed hamsters had lower weights and less fat than socially housed hamsters, but group-housed hamsters did have moderately smaller thymus glands, which may indicate stress. The authors emphasized the importance of keeping Syrian hamsters in stable groups—that is, that no animals be added to or removed from the group. Elidio et al. (2021) found that, compared to group-housed females, group-housed males were less aggressive toward each other and toward animal care technicians (e.g., vocalizing, attempting to bite the handler). Females grouped right after weaning were less aggressive toward each other and technicians than females grouped at 6–8 weeks of age. Single-housed females were least aggressive toward technicians; single-housed males were not included in this study.

While aggression is generally viewed as a negative experience, some research indicates that exerting dominance appears to be a rewarding experience for Syrian hamsters, although the welfare of the subordinate animal is likely reduced. For example, in one of the studies described earlier where male Syrian hamsters developed preferences for locations where they could interact with another hamster, preferences were significantly stronger for dominant compared to subordinate individuals (Gil et al., 2013). Additionally, in a study about the role of aggression in social interactions, single-housed ovariectomized female Syrian hamsters who attacked male intruders experienced various neural changes in the brain indicating that these aggressive interactions were rewarding to the female aggressors (Borland et al., 2020). The effects on males were not investigated.

In the wild, **naked mole rats (NMRs)** live in colonies of 25 to more than 300 individuals (Ragland et al., 2022; Yu et al., 2017). One study described their average colony size in captivity to be 11 (Yu et al., 2017), while another described colonies ranging in size from a single individual to groups of 200 (Smith & Buffenstein, 2021). NMRs are eusocial, meaning that only one female "queen" and up to three or four males reproduce, with all other individuals acting as workers. Offspring rarely leave the colony. NMRs are generally peaceful, but they can be very aggressive to individuals from outside their colony, or even to members of their own colony who smell different. For these reasons, laboratory staff must take extreme care when moving animals, because any escapee must be returned to their own colony. Additionally, staff must wear different gloves when handling individuals from separate colonies to avoid the transfer of latent

pheromones. If an outsider—or someone who just smells different—is introduced into a colony, they will likely be attacked, and this could trigger a colony-wide fight resulting in numerous injuries or even death. Individuals who survive an attack may nonetheless be ostracized; they are kept out of the nest and food areas and are usually found in a submissive posture inside the toilet chamber. These individuals are at risk of dying and must be removed from the colony. Even after removal, their outlook is often grim (they are known to stop eating), but sometimes they can be successfully paired with a different mole rat (Ragland et al., 2022; Smith & Buffenstein, 2021).

In the wild, **Damaraland mole rats (DMRs)** live in small to medium-sized colonies of 2–40 individuals, though captive colonies of up to 48 individuals have been described (Buffenstein et al., 2024). Just like NMRs, DMRs are eusocial, but they are more docile, and therefore reintroduction of escapees back into the colony is simpler. Buffenstein et al. (2024) indicated that their approach to reintroducing escapees is to remove all individuals from their tunnel system and introduce them to each other one at a time inside a novel rat cage. The animals are watched closely to make sure they do not start fighting, although they will "intensely" sniff each others' faces and genitals. Once all the individuals settle down together in the rat cage, they can be put back into their housing system.

Rabbits

GENERAL STRATEGIES

Wild European rabbits, who were selectively bred to produce the commonly used New Zealand white rabbits, live in large colonies (or "fluffles") of several hundred individuals. Fighting within colonies is rare, primarily because subordinate rabbits have space to remove themselves from dominant individuals. A subordinate rabbit seeking to de-escalate a situation must generally get at least 3 feet away from their aggressor (Gaskins, 2023). Captive rabbits sharing an enclosure require the same amount of available separation space, as well as a visual barrier or refuge to remove themselves from each other when needed. Rabbits who get along will share resources, groom each other, and rest together. Rabbits who are not getting along will not share the same space and will engage in chasing, biting, or wounding.

In a descriptive pilot project, Enser (2016) housed groups of rabbits in floor pens to reduce the use of conventional cages in an antibody production facility. Pens were permanently furnished with beds, platforms, and tunnels, while other items—such as toys and gnawing objects—were used in rotation (Figure 5). There were no welfare

Figure 5: Large groups of female rabbits can be housed in structurally complex floor pens without many welfare concerns, but males may need to be split into smaller groups once they reach sexual maturity.

concerns when housing females in groups of up to 20 individuals for up to 33 weeks of age, even when adding new adult animals to the established group. In contrast, group-housed males began exhibiting aggression at around 10–12 weeks of age, and the male groups were eventually split into trios. Pen size was not given, but images suggest rabbits were housed at high stocking density. The authors noted that they would try housing males in smaller groups of up to 10 individuals as a next step.

DiVincenti and Rehrig (2017) described the social behaviors of male New Zealand white rabbits when group housed in pens versus pair housed in cages. Pens measured 20 x 4 feet and contained four food dishes, four water bottles, and cardboard boxes

that served as shelters and visual barriers. Each pen housed six rabbits. When they were first placed in pens, rabbits showed a mixture of exploratory, affiliative, and agonistic behaviors, but no injuries occurred. Following the first day, one pen was calm and stable, while the other showed a mixture of affiliative (body-to-body and nose-to-nose contact) and agonistic (chasing without attacking) behaviors. Rabbits usually socialized in pairs, sometimes in trios, but never as a whole group. After 2 weeks, rabbits from the stable pen were split into pairs and rehoused in two conventional cages connected by a tunnel to determine if their interactions would change with restricted space and intentional pairing of familiar and compatible animals. The transition to pair housing in smaller cages resulted in a notable increase in mounting behavior and urine marking, with one pair requiring separation due to continued chasing and biting. Another three pairs of rabbits were formed on the day of arrival at the facility. All pairs were eventually separated due to persistent aggression and incompatibility, with researchers noting that subordinate animals were remaining motionless in the corner, except to escape chasing or attacks. It was also noted that subordinate pair-housed rabbits attempted to flee from aggression but were unable to retreat or demonstrate submission due to limited space. The additional space of the pens may have allowed for more affiliative behaviors and greater success in defusing aggression.

Valuska and Mench (2013) evaluated whether enclosure size affected the behavior of female New Zealand white rabbits meeting for the first time. Females unknown to each other were marked with the urine of the same male on their foreheads and then placed in a small (2.5 x 4 x 2 ft) or large (4 x 4 x 2 ft) enclosure. Enclosures were custom built with plywood and were divided in half using vertical PVC bars (0.5 in. diameter) spaced 2 inches apart; this allowed rabbits to groom or bite each other but not pursue or attack one another. Half of the pairs were introduced to each other in the small enclosure first and the large enclosure 72 hours later, and the other half were introduced in the opposite order. During their first meeting, pairs in the large enclosure engaged in significantly less aggression and trended toward more affiliative behavior and shorter latency toward affiliative behavior compared to pairs in the small enclosure. Interestingly, during their second meeting, each pair behaved similarly to how they behaved during their first encounter, which meant that the results were reversed: pairs in the small enclosure showed less aggression. The authors concluded that larger enclosures were effective at reducing aggression in pairs of rabbits meeting for the first time, but that during reintroductions, prior experience may matter more than enclosure size.

Building on the idea that a larger space for initial introductions is important, Marshall et al. (2017) described their facility's approach (still in use as of this writing) to

grouping female New Zealand white rabbits when they first arrive at their facility. Briefly, rabbits remain in their cardboard transport boxes as they are placed into a large (3 x 4 ft) dog exercise pen containing litter boxes (used rodent transport boxes), tunnels (made from urine descaler buckets), floor toys, huts, Carefresh bedding, and water bowls (Figure 6). Staff monitor the animals closely from within the room for 2 days, and aggressive individuals are split into pairs or trios. Once groups are established, they are monitored via internal "bunny cams." The authors concluded that establishing social groups in a spacious environment under close supervision allows them to see which individuals have an affinity toward one another. Eventually, those animals can be housed in pairs within conventional cages connected with a tunnel or they can remain in a large social group.

Figure 6: Marshall et al. (2017) established stable groups of female rabbits by placing newly arrived animals who were still within their cardboard transport boxes into a large pen under close supervision.

Thurston et al. (2018) described methods that were most successful at their facility for pair housing New Zealand white rabbits in a "doublewide" cage consisting of side-by-side standard cages with the middle partition removed. The protocols, which were published in the *Journal of Visualized Experiments*, are accompanied by a step-by-step video. Methods include capitalizing on rabbits having undergone the stress of transport together ("stress bonding"), urine marking, providing a neutral territory

(a clean cage that is new to both rabbits), and providing sufficient resources so that rabbits aren't forced to share them. When pairing females previously unknown to each other, their foreheads are marked with male urine, since this is believed to signal to the rabbits that they belong to the same breeding group. Both females are then placed in a neutral doublewide cage that contains a minimum of two low-value items (those that can be moved or chewed, like chew sticks, balls, or bells) and two high-value items (those that can be destroyed or eaten, like stuffed cardboard tubes or foraging toys), two hiding opportunities (e.g., hut, box, or perch), two piles of loose hay, and two unique access points for food and water. Pairs are monitored continuously for the first hour after pairing, so that staff can intervene in case of fighting or wounding. Pairs are deemed to be stable if they are observed engaging in clear dominant and submissive behaviors, eating and drinking, resting together, and/or allogrooming. If this is the case, observations during the following week can be decreased to a daily 10-minute period. Stability of rabbit pairs is documented using a social introduction log, in which affiliative, neutral, and aggressive behaviors are tracked. A template log is provided with the article. Additional enrichment interventions may also be useful when agonistic behaviors are observed. Variations of this protocol are provided for young female sibling and non-sibling pairs, young females with their mother, young male siblings, and adult males paired by the vendor prior to arrival.

Santos et al. (2023) also described a successful pairing process for 2- to 5-kilogram adult female New Zealand white rabbits with no prior social housing experience. Rabbits are marked with their future mate's urine on the nose, feet, and hindquarters and immediately placed in a novel Euro-style cage with the divider panel removed. Pairs are kept together and continuously observed for 20–30 minutes, then separated. Time together is increased each day if no aggression is observed. A towel is used to redirect or separate fighting rabbits. Pairs are left together overnight only after there has been no aggression for several continuous days of observation. The authors have been using this method to successfully pair adult female rabbits used in pharmacology and toxicology testing for approximately 5 years.

Social preferences may vary according to biological and environmental variables. Dal Bosco et al. (2020) conducted two experiments to test the preference of female New Zealand white rabbits for social contact. In the first experiment, nulliparous rabbits (those who have never given birth) were housed in groups of four within enclosures that allowed for seclusion or social contact. Enclosures consisted of a central social area (6 x 2.5 ft) containing a food hopper and water bottle; on each end were a pair of individual seclusion areas (1.2 x 0.8 ft) accessible through a push door. The rabbits in this experiment spent roughly half of their time in the seclusion areas. In the second

experiment, four rabbits and their litters were housed in an enclosure consisting of a group area (7.8 x 2.6 ft) attached to four individual spaces (3.3 x 2 ft) with nest boxes (2 ft x 0.7 ft). Each adult had access to her own individual space and nest box via an RFID microchip. The group area contained two feeders, four drinkers, two hay dispensers, and an elevated platform, and each individual space contained an additional feeder, drinker, hay dispenser, and elevated platform. The rabbits in this experiment tended to stay in their individual nesting areas roughly 72% of the time. The authors concluded that preference for contact with conspecifics may change based on the presence of kits and/or location of food and water: Time in the social area may have been higher in the first experiment because that was the only space where food and water were available. However, it is also worth noting that the social areas in both experiments were barren, and that the open space without refuge likely made those areas unappealing to rabbits. In the second experiment, the presence of a nest box in the individual space may have further enticed rabbits to spend more time there compared to the larger open space.

A number of studies have investigated strategies for providing social housing to breeding females in commercial farm settings. While methods and approaches differed substantially between these studies, two findings were consistent: Aggression is highest on the day female rabbits are grouped or regrouped after a period of separation and subsides after a few hours; and if rabbits are regrouped after a period of separation, aggression is lower if they are placed with their previous mates versus unfamiliar rabbits (Andrist et al., 2012; Buijs et al., 2015, 2016; Munari et al., 2020; Zomeño et al., 2017).

Attempts have been made to offer some form of social companionship to single-housed rabbits, including through temporary playpens. In a descriptive pilot project, five single-housed, pregnant New Zealand white rabbits in a reproductive toxicology program were provided group exercise sessions three times per week for 30 minutes at a time (Holmes et al., 2017). The exercise arena (of unspecified size) contained toys, tunnels, and hay. No adverse clinical effects were detected from these social sessions, and reproductive performance was marginally improved compared to a control group. However, the rabbits exhibited aggression whenever they were regrouped for the exercise period (though it decreased with each new session—from almost continuous aggression at the beginning to subsiding within the first 5–10 minutes in later sessions). During the sessions, rabbits showed a mixture of positive (allogrooming, nuzzling, lying next to each other) and negative (thumping, chasing) behaviors. Ultimately, the need for staff to frequently intervene to prevent injurious fighting led to discontinuation of this type of temporary social grouping.

Testing whether mirrors could be used as stand-ins for conspecifics, Edgar and Seaman (2010) provided single-housed New Zealand white rabbits of both sexes with a mirror for 1 week. The mirror's dimensions (1 square foot) and placement were such that rabbits could avoid their reflection if they chose to do so. There were some behavioral changes when the mirrors were present, such as decreased grooming and increased investigatory behavior. However, these behavior changes are not sufficient to indicate improved welfare, particularly in comparison to the behavior changes observed in the presence of a social companion. Similarly, Mastellone et al. (2019) conducted an experiment comparing the behaviors of farmed male New Zealand white x California rabbits who were either isolated, isolated with mirrors, or separated by a mesh partition allowing for visual and olfactory contact. Rabbits who could see and smell neighboring rabbits spent more time being active, gnawing, stretching, and sniffing. Rabbits housed with mirrors spent more time grooming and less time in their shelter than rabbits who were housed in isolation without mirrors. There were no differences in cortisol levels between groups. Taken together, these results suggest that mirrors may provide some stimulation for rabbits, but do not match the effects of stimulation from social companions.

CHALLENGES SPECIFIC TO CERTAIN EXPERIMENTAL MANIPULATIONS

Animals with bandages or sutures are commonly outfitted with an Elizabethan collar; while these collars prevent animals from accessing their wounds, they also prevent them from engaging in other important activities, such as self-grooming,

Figure 7: In Bartley and Johnson (2019), rabbits outfitted with human infant pants and kept with their social companion after surgery had equally good healing outcomes, but markedly better pair maintenance compared to rabbits outfitted with a collar and separated for 10 days.

coprophagy, and a variety of social behaviors. To avoid single housing intact male New Zealand white rabbits after two femoral angioplasty procedures performed a month apart, Bartley and Johnson (2019) trialed the use of human infant pants instead of collars (Figure 7). After each surgery, rabbits were either outfitted with pants immediately after surgery and re-paired just after recovery from anesthesia, or outfitted with a collar and single housed for 10 days before re-pairing. The pants were sized 0–3 months (designed to fit infants weighing 5–7 kg and measuring 24–25 in.) and made of 100% cotton, with full elastic waistbands. A 2- to 3-inch slit was cut to expose the rabbit's tail and genitals for toileting. The authors found the pants to be much more effective at maintaining pairs than collars, with a re-pairing success rate of 82% and 97% after first and second surgeries, respectively, compared to 5% and 0% for collars. There were no differences in self-mutilation (minimal) or wound healing (completely healed by the time protection was removed) between the two treatments. About two-thirds of the pants needed replacing within the 10-day post-operative period after the first surgery, compared to about one-third during the post-op period following the second surgery; the authors attributed the significant decrease in pant failure rate to the team's learning curve regarding proper fitting and to the rabbits' habituation to wearing them.

KEY TAKEAWAYS: SOCIAL HOUSING

- Most species of **rodents** and **rabbits** are highly social, and efforts should be made to socially house animals with compatible cagemates as much as possible (including after surgery and during experimental procedures). Social companionship is widely regarded as the single most important feature of a captive environment and has the greatest influence on animal welfare.

- Sometimes, social animals are single housed in order to avoid the experimental variability introduced by the social companion. However, this way of thinking fails to account for the experimental variability introduced by *isolating* social animals, which has many downstream effects on physiology, behavior, and translational validity.

- Aggression is a valid welfare concern. It is important for cagemates to get along, as aggression among cagemates can have negative welfare impacts, just as social isolation can. The restrictive and unnatural conditions under which **rodents** and **rabbits** are conventionally held in laboratories likely triggers higher levels of aggression. It may not be possible to eliminate aggression without making fundamental changes to the way these animals are housed. But even within conventional enclosures such as shoebox cages, steps can be taken to decrease aggression and promote safe social housing.

- **Mice**—even males—can be successfully housed together. Best practices include grouping mice at a young age (i.e., at weaning and up to 4–5 weeks of age), maintaining stable social groups (i.e., avoiding regrouping), transferring used nesting material (but not bedding) at cage changing, and avoiding the use of corncob bedding or individually ventilated cages. Adding partial cage dividers that split the cage into distinct sections can help de-escalate aggressive interactions.

- **Rats** are less likely to demonstrate aggressive tendencies than mice and should be housed in social groups. Wire mesh partitions may be useful during introductions, but full physical contact (i.e., without mesh partitions) is necessary to achieve social buffering benefits (e.g., reduced anxiety) when the animals undergo stressful experiences. Adolescent rats may be particularly sensitive to social isolation; single housing during this period should be avoided. Refinements exist that allow rats to be socially housed even when wearing cranial implants or telemetry devices.

- **Chinchillas, degus, prairie voles,** and **guinea pigs** benefit from social housing. Forming groups as early as possible and keeping the groups as stable as possible (avoiding adding or removing animals) is often advantageous.

- **Hamsters** may tolerate stable social groups, and group housing from a younger age may help to reduce aggression, but hamsters (particularly females) may be least stressed when housed individually.

- **Naked mole rats** live in large social communities, but they are very aggressive to community outsiders, or even community members who smell strangely; caretakers, therefore, must use extreme caution when handling, removing, and reintroducing animals to the colony.

- **Rabbits** can be successfully housed in pairs or groups, and having access to a compatible social partner may provide benefits such as improved thermoregulation and social buffering in stressful situations. Aggression is a welfare concern when socially housing rabbits, particularly in males or does with kits. When establishing new pairs of rabbits, helpful methods may include marking females with male rabbit urine, stress bonding (i.e., pairing animals who have undergone a mildly stressful event together, such as transport), providing and regularly rotating a variety of environmental resources, using larger cages or pens, and using clean cages during initial introductions. Stable social groups should be maintained, especially for male rabbits who can be severely aggressive following regrouping. Socially housed rabbits benefit from separate areas or nest boxes where they can escape aggression. Human infant pants can be used instead of Elizabethan collars to allow for social housing of rabbits following surgery.

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Environmental Enrichment

What Is It, And Why Does It Matter?

Increasing the complexity of the home environment can have extensive welfare benefits for rodents and rabbits housed in laboratories. "Enriching" the environment in a biologically meaningful way can help rodents and rabbits feel safe and mentally stimulated. Resources added to the home cage can make the space more usable, but it is also important that it not be so crowded as to impede movement. Rodents housed in more complex environments have lower incidence of disease, lower disease severity, and live longer than animals living in typical shoebox cages (Cait et al., 2022)—all factors that are also highly relevant to their use in research.

Altering the home environment can have important impacts on welfare and behavior, which may raise concerns about altering experimental outcomes. However, there is a growing body of evidence that providing environmental enrichment in rodent cages does not adversely impact variability of experimental outcomes (André et al., 2018; Cait et al., 2022; Kentner et al., 2021). For example, the addition of nesting material or shelters may change some study parameters, but it does not increase variability of results; this means that these important resources can be provided without fear that they will compromise the validity of experiments (André et al., 2018) or necessitate the use of more animals to detect statistically significant differences. Indeed, to obtain valid physiological data, it may be *necessary* to provide nesting material (Maloney et al., 2014). In one study where CD1 mice were provided 10 grams of paper nesting material, major clinical parameters were unaltered, but indicators of welfare were improved (mice had reduced piloerection and fecal corticosterone metabolites, which are indicative of stress; Brochu et al., 2018). Moreover, providing environmental enrichment has been shown to not only benefit animals but also reduce compassion fatigue for laboratory personnel (LaFollette et al., 2020).

While there are many definitions of the term *environmental enrichment*, we define it here as resources or stimuli that improve measures of animal welfare and promote the expression of species-appropriate behavior (Ratuski & Weary, 2022). It is important to recognize, however, that the term is often a misnomer: Calling something "environmental enrichment" implies that it results in an environment that exceeds basic requirements, possibly even providing "luxuries," but that is often not the case (Newberry, 1995). A typical laboratory cage is poorly provisioned. It may contain components—such as nesting material or a shelter—that are commonly referred to as enrichment yet merely serve to meet the animals' most basic needs.

In this book, we use the term *environmental enrichment* to broadly describe various resources that can be provided to animals; however, in the interest of striving for high standards of animal care through ongoing refinement, we encourage readers to avoid thinking of cages containing any one of these components as "enriched." We also encourage critical consideration of the biological relevance and desired welfare outcomes when introducing a new resource, since not all additions to an environment will be beneficial for welfare, and what is suitable for one species may not be appropriate for another. When adding a novel item into a cage—especially when space is limited, such as within standard shoebox cages—it is important to consider what purpose the item is meant to serve, and whether the item will be biologically relevant or beneficial to the animal.

Here, we first provide a brief high-level introduction to the different categories of enrichment used for rodents and rabbits, before summarizing new evidence in the more detailed sections that follow.

Nesting material: The range of temperatures at which an individual feels comfortable and does not need to expend extra energy to keep warm is called the "thermoneutral zone." In humans, the lower end of the thermoneutral zone is 70 °F (21 °C), but rats need 82 °F (28 °C) and mice need 86 °F (30 °C) to be comfortable (Gordon, 1990, 2012). This poses a problem, since rodent housing rooms tend to be kept at temperatures that are comfortable for humans. For rodents, living in these colder temperatures results in important changes to physiology and metabolism, as animals expend more energy trying to stay warm. For example, compared to mice housed at 86 °F, mice housed at 68 °F (20 °C) have significantly higher heart rates, higher daily caloric intake, and altered brown fat content (indicative of cold stress; Maher et al., 2015). Cold stress negatively impacts both animal welfare and the scientific value of rodents as a model for human disease (Maloney et al., 2014); indeed, "available data suggest that the cold stress to which laboratory mice are ubiquitously subjected profoundly affects mouse physiology in ways that impair the modeling of human homeostasis and disease" (Karp, 2012).

Providing rodents with nesting material can help them stay warm and consume less food, and nest building allows them to engage in a natural behavior while exerting some control over their home environment (Gaskill, Karas, et al., 2013; Gaskill & Garner, 2014).

Huts, tunnels, and nest boxes: Shelters can help rodents and rabbits feel safe by providing physical cover when they feel the need to hide from perceived threats or bright lights. Mice do not typically use huts for sleeping but will retreat into them

when frightened (Sherwin, 1996). Shelters can also decrease the expression of stereotypies (Callard et al., 2000; Leach et al., 2000; Würbel et al., 1998).

Feeding and foraging enrichment: Rodents in the wild spend a significant portion of their day searching for food; accommodating this natural behavior in a laboratory setting by providing foraging opportunities can increase activity and reduce boredom (Johnson et al., 2004). Rodents may actually prefer to "work" for their food, as long as the work demand is not too high (Carder & Berkowitz, 1970). Feeding in creative ways that require the animals to actively search for food can be stimulating. A varied diet (e.g., seeds, fruits, vegetables, grains) of different textures, tastes, and delivery methods may appeal to rodents and rabbits alike, although care should be taken to ensure that dietary changes do not compromise ongoing research or animal health (Jennings et al., 1998).

Environmental complexity: In many research studies, animals are simultaneously supplied with a variety of enrichment items, such as nesting material, shelters, running wheels, treats, or other objects. Often these studies cannot disentangle the effects of any particular resource over another, so conclusions are discussed in relation to overall increased environmental complexity. The simultaneous provision of multiple types of enrichment may be beneficial in and of itself, because it allows animals to make choices and engage with their environment in ways that align with their varying needs (Špinka, 2019).

Auditory enrichment: Auditory enrichment (music or background noise) has the potential to reduce monotony and mask sudden noises that could be frightening or stressful to animals, while also improving the work environment for facility staff. However, music can affect physiology and behavior; as such, playing music or radio in research facilities should be regarded as a potential confounding variable in studies, given the varying effects of different volumes, sound frequencies, and content of radio programs (Patterson-Kane & Farnworth, 2006).

Auditory enrichment has been studied rather haphazardly. To help make use of music or sounds in a more thoughtful way for each species, Snowdon (2021) proposed a conceptual framework that is based on knowledge of (1) the species' natural methods of communication and (2) how different musical structures influence emotional states. For example, elephants and baleen whales communicate in the infrasonic range, while rodents and bats communicate in the ultrasonic range; therefore, limiting sounds to the narrow human range may be irrelevant or unhelpful. Even if ultrasounds were included in an audio track, however, many speaker systems would

not have the auditory range to reproduce them. Moreover, while many studies on auditory enrichment tend to compare music genres (e.g., classical versus rock), there is great variation within each genre. The desired effect on the animal—calming versus arousing, for instance—requires consideration of the tempo, pitch, rate, amplitude, harmony, and attack speed of each musical piece (Snowdon, 2021). To achieve welfare goals, it may be necessary to carefully construct sounds tailored to the particular species' perceptual range rather than playing existing music made for humans (Kriengwatana et al., 2022).

Finally, a word of caution: While individual animals can choose whether to interact with physical enrichment such as shelters or chew toys, they generally cannot tune out auditory enrichment if they dislike it or simply want a break from it. For this reason, auditory enrichment may be most effective if provided in a manner that allows animals to have control over it. For example, one facility provided white-faced saki monkeys with a variety of sounds that the monkeys could turn on by entering a tunnel-shaped structure equipped with sensors and speakers (Piitulainen & Hirskyj-Douglas, 2020). The device played sounds only when a monkey entered the tunnel. This concept of "on-demand" auditory enrichment could likely be adapted to rodents and rabbits.

Summaries of Current Refinement Research

Mice and Rats

The abundance of research on environmental enrichment for mice and rats has led to many review articles that attempt to synthesize the findings. Simpson and Kelly (2011) summarized literature on environmental enrichment for rats and advocated social housing, larger cages, and a combination of both social and physical elements in the cage. Baumans and Van Loo (2013) discussed strategies for mice and rats, recommending social housing, nesting material, chewable items, and opportunities for foraging. Bayne (2018) reviewed enrichment for mice, recommending nesting material and nest boxes or shelters. Others endorsed a wider variety of enrichment options for mice; for example, Lewejohann et al. (2020) recommended larger cages, nesting material, gnawing substrates, burrowing opportunities, and positive reinforcement training. Pritchett-Corning (2019) recommended a minimum of bedding, social partners, nesting material, and shelters in "standard" rodent cages, while advocating a shift toward semi-naturalistic housing (i.e., with substantially more space than is currently available in typical rodent cages and with more opportunities to express natural behaviors).

In an effort to synthesize this literature even further, Ratuski and Weary (2022) conducted a meta-review (a review of review papers) on environmental enrichment for laboratory rodents. They found that the environmental enrichment most often recommended in the review articles were social housing, foraging opportunities, nesting material, huts or nest boxes, and larger cages. They also noted that the authors of literature reviews commonly outlined their own perceived risks and requirements of environmental enrichment (e.g., voicing concerns about practicality, effects on experiments, or availability of resources) that may represent more implicit barriers to implementing enrichment. Overall, Ratuski and Weary advocated the use of more specific and value-neutral terms to describe environmental modifications and how they might affect the welfare of the animals.

Systematic reviews and meta-analyses (reviews that analyze and combine data from all relevant empirical studies) of environmental enrichment have also been conducted in recent years. In one example, Cait et al. (2022) reviewed and analyzed 214 studies to test whether mortality and the severity of certain diseases known to be exacerbated

by chronic stress—anxiety, cancer, cardiovascular disease, depression, and stroke—were higher in conventional rodent housing (shoebox cages with minimum mandated cage contents) than in better-resourced cages. They found that conventional housing increased the severity of all diseases assessed, in addition to increasing mortality rates. For animals housed in standard cages, this raises concerns over both welfare and the quality of data collected. The authors also noted that data variability was not increased in enriched housing conditions. In another systematic review of neuroscience research (125 mouse studies and 156 rat studies), Kentner et al. (2021) echoed this finding: Results from rodents housed with enrichment are not more variable than those from rodents housed in standard conditions. In a follow-up publication that reviewed and analyzed 232 articles, Cait et al. (2024) tested the hypothesis that providing more resources would provide health benefits in a dose-dependent manner. The hypothesis was supported: There was a steady decrease in morbidity with each additional type of resource added to the cage. The maximum number of resource types they were able to test was five, and because there was no evidence of a plateau within this range, the authors recommend that even more resource types should be provided to further improve rodent health and welfare. Mieske et al. (2022) conducted a systematic review of the effects of enriched environments on various parameters related to animal welfare, dividing enrichment into three categories: social partners, additional space, and objects. They found that most studies used a combination of all three, and that enrichment tends to increase social behavior, motor function, affective states, and cognition, while decreasing abnormal behavior.

Mice

NESTING MATERIAL

Both male and female mice actively use and benefit from nesting material, even when other shelters are provided (reviewed by Olsson & Dahlborn, 2002). The *type* of nesting material matters to mice. Male and female C57BL/6 and BALB/c mice prefer facial tissue paper or paper towels over crinkle paper (Enviro-Dri) and generally prefer paper-based nesting materials over materials made from less-processed wood (Van de Weerd et al., 1997). The type of nesting material also matters for nest complexity. Obermueller et al. (2021) assessed the nest-building behavior of single- or pair-housed male and female C57BL/6J mice (who tend to be poor nest builders) and BALB/c mice (who tend to build high-quality nests) when provided various types of nesting material: two cotton Nestlets, four “cocoons” (short cotton fibers), 4 grams of wood wool, 8 grams of compact crinkle paper, or 8 grams of compact softwood shavings. Pair-housed C57BL/6J mice achieved better quality nests compared to single-housed

mice of that strain. Both strains built the most complex nests with cotton Nestlets compared to all other materials; wood wool nests received the lowest nest quality scores. In contrast, MacDuff et al. (2019) found that their two in-house strains (F1 c/c and Bx c/c) built better nests with crinkle paper compared to cotton Nestlets. Bárdos et al. (2022) gave 25th-generation, captive-born offspring of wild *Mus musculus* simultaneous access to long blades of hay, crinkle paper, and nonfibrous cotton (MultiFit small rodent cotton bedding). Mice used hay in the greatest proportions (76%), followed by crinkle paper (21%) and then cotton (3%). Nests using higher proportions of hay were of higher quality, likely because hay provides more structure. Finally, Rodgers et al. (2020) compared two cotton Nestlets to three cotton dental rolls (for use in human dentistry) in dozens of cages with mice of various strains. Neither material yielded good nests (i.e., with a mean nest quality score of at least 3 out of 5) on any day during the trial. Technicians setting up clean cages initially preferred distributing the cotton dental rolls. Use of this material was immediately discontinued, however, when the technicians discovered that several of the mice had gotten limbs tangled in the dental rolls, resulting in limb amputations.

A series of experiments by Gaskill and colleagues demonstrated how nesting material is used by mice to control the temperature within the microenvironment of the cage. In one study, C57BL/6J mice were housed in systems consisting of three cages, each maintained at a different ambient temperature: 68 °F (20 °C), 77 °F (25 °C), or 86 °F (30 °C). As predicted, mice chose to spend the most time in the 86 °F condition and the least time in the 68 °F condition. However, when mice were provided with nesting material, time spent in the 68 °F condition doubled. When mice could not choose to avoid the 68 °F condition, their nest quality increased, while nests in the 86 °F condition were lower quality (i.e., cup-shaped or flat, rather than dome shaped) because mice did not require as much insulation in this environment (Gaskill et al., 2011).

In another experiment, researchers compared C57BL/6, BALB/c, and CD1 mouse preference for cages with warm ambient temperatures ranging from 68 to 95 °F (20–35 °C) against their preference for crinkle paper nesting material (0–10 g). Females preferred warmer temperatures than males, but all strains and sexes preferred to sleep in temperatures between 79 and 84 °F (26–29 °C). While there were differences in the thermoregulation strategies of the various strains, they found that mice did not begin to spend significant amounts of time in the nesting material cage until at least 6 grams of material was provided. They concluded that under normal laboratory conditions, mice should be provided with at least 6 grams of nesting material, but up to 10 grams may be necessary to alleviate thermal stress, especially in ventilated cages (Gaskill et al., 2012).

A follow-up experiment housed C57BL/6, BALB/c, and CD1 mice with or without 8 grams of crinkle paper material for 4 weeks. They found that mice with nesting material ate significantly less food than those without nesting material, and mice who built higher quality nests (more dome-like) conserved more heat. CD1 mice with nesting material had higher body weights than controls, and male mice with nesting material had less T_4 concentration (a proxy for metabolic rate) in blood samples, indicative of better thermoregulation. Females appeared more thermally stressed than males, suggesting that 8 grams of nesting material may not be sufficient for females housed at 68 °F (20 °C) (Gaskill, Gordon, et al., 2013). Another study by Maher et al. (2015) came to a similar conclusion regarding single-housed females at 68 °F: Providing 10 grams of crinkle paper nesting material only slightly normalized measures of cold stress. Additional strategies should be considered to keep mice warm, such as providing social partners, more nesting material, or higher room temperatures.

Nesting material offers other benefits: For example, Moreira et al. (2019) gave breeding pairs of BALB/cJ and Swiss Webster mice 3 grams of cotton and eight pieces of disposable polypropylene hairnets as nesting material. Provision of nesting material promoted more contact between parents and pups and increased the time dams spent licking and grooming their pups, which can aid their development. In another facility, adding 10 grams of crinkle paper and cotton Nestlets to the cage reduced aggression in male CD1 mice by 27% (Veness et al., 2023). Some facilities only provide a small amount (< 6 g) to enable easier viewing of the animals during health checks, but mice require more nesting material to form high-quality nests and maintain thermoneutrality. Assessing daily census and health report data from a variety of mouse strains, one study found that provision of 6 grams of crinkle paper did not obstruct the ability of animal care staff to identify sick or dead mice during routine cage-side checks (Burlingame et al., 2021). Johnson et al. (2017) provided 0, 6, or 12 grams of crinkle paper to C57BL/6, BALB/c, and CD1 mice housed at 68 °F. There was no difference in food consumption between treatments, but mice provided with 12 grams of nesting material maintained a more positive energy balance (energy derived from feed minus metabolic energy output) and greater body weight than mice with 0 or 6 grams of material.

Mice who are older or otherwise weaker have trouble processing cotton Nestlets and often end up just sitting on top of them. To help them out, Evans (2016) placed a few Nestlets in a blender for 5 seconds, resulting in “a billowy, completely shredded fluff.” Within a short time, mice who had been unable to use cotton Nestlets were burrowing into the fluff (Figure 1). An alternative to cotton Nestlets are Bio-Serv’s paper nesting sheets. Froberg-Fejko (2010) recommended placing these sheets into the wire lid of

Figure 1: Shredding cotton Nestlets in a blender allows mice who have trouble processing them to use the fluff as nesting material (Evans, 2016).

the cage as a way of monitoring mouse health: A healthy mouse will typically pull the paper into the cage and use it to construct a nest; if the sheet remains untouched, this may indicate a health concern that warrants attention. Another product, CellPad, allows for compressed cellulose nesting material to be suspended from the cage lid. CD1 mice provided with a CellPad actively broke it down and incorporated the materials into their nests (Burbidge et al., 2023). To reduce staff time and ensure that all cages are provided with the same amount of nesting material, the material could be processed through a bedding chipper in conjunction with bedding, so that both resources are distributed simultaneously. However, Robinson-Junker et al. (2017) found that processed crinkle paper had a negative effect on the integrity of the nesting material, which resulted in poor-quality nests. This was especially true for C3H mice, who built poorer-quality nests than BALB/c mice when the material had been processed, but not when it was intact. The authors also found that unprocessed nesting material can be mixed in with the bedding, as this encourages mice to sort through the bedding to pick out the material and incorporate it into their nest; sorting may be beneficial to mouse welfare because it is a species-typical behavior.

Mice with surgically implanted head plates may become tangled in long or fibrous nesting material. To assess this risk and potential solutions, Windsor and Bate (2019) provided a variety of nesting materials to mice with external head plates. They compared facial tissues, Rodent Rolls (compressed, short-fibered material), Pure Comfort White (soft shredded paper material), and short paper shavings. Tangling was only ever observed in cages provided with facial tissues. Nests constructed with Pure Comfort White were consistently high quality and posed no safety issues. Short paper shavings and Rodent Rolls were also deemed safe, but nests were of lower quality with these materials. In a follow-up study, mice with head plates were provided with Pure Comfort White, cotton Nestlets, or a combination of the two (Windsor, 2021). Nests constructed with combined materials had the highest nest quality scores, while cotton Nestlets resulted in the lowest nest quality scores (although the difference was not statistically significant). No tangling was observed in any of the treatments, indicating that these are safe to use with mice after head-plate surgeries.

HUTS, TUNNELS, AND NEST BOXES

Mice have preferences for different styles of shelters. Tunnels (or “tubes”) are convenient to provide in the home cage because they can double as a tool for handling mice (see chapter on **Human-Animal Interaction**); however, there is evidence that handling tunnels may not be adequate as a shelter. Burn and Popat (2021) assessed sheltering behavior when C57BL/6 male and female mice were provided a

clear handling tunnel, a clear handling tunnel with an additional cardboard tunnel, or a clear handling tunnel with an additional amber-colored dome ("igloo"). Mice with the igloo sheltered three times more than mice with only a handling tunnel, potentially because the igloo enabled more mice to shelter together. Cardboard tunnels were not popular for sheltering but were sometimes used for gnawing. Mice were sometimes observed nesting in the igloos, while they were never observed nesting in the handling or cardboard tunnels. In an observational study, C57BL/6 female mice chose either blue or amber igloos over red igloos (Gjendal et al., 2018). However, mice were given access to the igloos for only 11.5 hours, which may not be long enough for them to establish nesting sites and form a true preference between the available options. Approximately 41% of mice in this study were observed inside a hut for 1 minute or less during the observation period, which could indicate that the observation periods were too short, or that shelter access is more important for situations when mice feel the acute need to hide (rather than nesting inside the huts).

Providing shelters can improve welfare without negatively impacting research outcomes (and can, in fact, positively impact such outcomes). In a study by Oatess et al. (2021), trios of male or female C57BL/6J mice were assessed for a range of physiological, neurological, and behavioral outcomes when housed in cages with nesting material and with or without a red acrylic tunnel. There was no notable increase in aggression, no difference in variability of results between treatments, and no significant difference between treatments for most measures. The tunnels even slightly decreased anxiety in the open field test, and male mice housed with tunnels had increased body weights, potentially due to better thermoregulation. Swetter et al. (2011) group housed male or female BALB/cJ mice in one of four environmental conditions: unfurnished cage, cage containing an igloo shelter, cage containing an igloo shelter with a running wheel, or cage containing an igloo shelter with one of eight additional plastic objects (e.g., short tunnel or hollow cube with multiple entries). The presence of an igloo did not increase variance in any experimental outcome and actually decreased variance in some measures. Both the igloo alone or the igloo with a novel object increased longevity and decreased aggression compared to the unfurnished cage. In males, the igloo with an attached running wheel increased aggression, but this was still lower compared to aggression in unfurnished cages; indeed, male mice in barren cages were prematurely euthanized more often than mice in any other treatment due to aggression-related injuries.

To cut down on expenses related to purchasing new shelters from commercial vendors, clear household glass bottles or jars donated by staff can be provided as

shelters. Rossi (2017) demonstrated that various solid-bottomed glass bottles or jars (e.g., pasta sauce or jelly jars) are readily used by mice and even tend to be preferred over plastic igloo huts (Figure 2). The glass shelters were often used as a nesting site by nursing dams and, if placed upright, were particularly effective at keeping nests with pups warm and dry in the event of a cage flood. Glass can be more easily sanitized than plastic and may be a better choice if researchers are concerned about the risk of animals ingesting plastic. A glass container may also allow for quicker and less disruptive transfer of animals at cage changing, particularly if moving a nest full of pups. Clear glass shelters allow for easy observation of animals during health checks, but the animals may prefer darker glass shelters if given the option. Collier (2010) designed and tested various red-tinted glass shelters for use in rodent metabolic cages. The most successful design for mice was a yurt-like structure with double walls and a vacuum at the top to aid body heat retention (Figure 3).

Figure 2 (left): According to Rossi (2017), solid-bottomed glass bottles or jars are readily used as shelters by mice and even tend to be preferred over plastic igloo huts. Figure 3 (right): For use in mouse metabolic cages; Collier (2010) found that a yurt-like shelter with double walls and a vacuum at the top to aid body heat retention worked well.

STRUCTURAL ITEMS

Access to a running wheel, even for as little as 1 hour per day, can benefit mice. In one study, single-housed male and female C57BL/6 mice were given access to a running wheel for 0, 1, 3, or 12 hours per day for approximately 8 months. Exercise, regardless of length of access to the running wheel, resulted in reduced weight gain compared to no exercise, even though mice with longer running wheel access ate more food. All mice with running wheel access demonstrated improved motor function and reduced anxiety in an open field test (Robison et al., 2018). However, running wheels may have different effects depending on mouse strains. In a case study on male FVB;129/Hemc mice, the presence of a running wheel in the cage resulted in pronounced stereotypic circling or route-tracing behavior, with the authors cautioning that running wheels may trigger abnormal behavior or brain biochemistry in mice of this strain (Leduc et al., 2017). Mice of other strains in this colony (BALB/cCrl, FVB/N, and C57BL/6) were not observed circling. Burbidge et al. (2023) outlined pros and cons of wheels as enrichment: Wheels provide exercise opportunities, are easily sanitized, and can be cost effective over the long term, but they require a lot of space within a shoebox cage and may not be appealing to all animals. Acknowledging that there may be economic and technological barriers to widespread implementation of running wheels for mice, Grigsby et al. (2024) showed that the "Dependable, Simple, Cost-effective (DSC)" 3D-printed open-source running wheel they designed is used similarly and is as effective at measuring wheel running behavior in mice as other commercially available wheels.

A variety of climbing structures or other cage furniture have been tested with mice. Õkva et al. (2010) compared three wooden structures (V-shaped ramp, enclosed nest box with two openings, and "stairs" consisting of several connected aspen blocks) with male C57BL/6 mice. Compared to mice in barren cages, mice housed with a nest box for 3 weeks or with stairs for 2–4 weeks showed decreased anxiety in the elevated plus maze test. Vogt et al. (2020) recycled wire cage lids to make bridge-like climbing structures for mice, noting benefits such as cost effectiveness and good visibility of the animals during health checks. Although mice made use of the provided structure, there were no differences in levels of stress hormones or in behavioral tests of locomotion, neophobia, anxiety, or sociability. Notably, the provision of the climbing structures did not increase variability of data in male or female C57BL/6 NRj mice, nor did it increase aggression among male mice. Dean et al. (2018) proposed autoclavable plastic cable ties hung on the wire lid of the cage as an inexpensive structural enrichment for mice. Preliminary research indicates that mice do engage with cable-tie swings in their cage, but different strains may use them differently, and more research is needed to demonstrate welfare benefits.

In an assessment of items that promote natural gnawing behavior to prevent malocclusion, Lopez Juaristi (2019) provided male and female mice of an undisclosed strain with one of five items for 2 weeks at a time: aspen balls, aspen chew sticks, flat birch chew sticks, small aspen bricks, and popsicle sticks. Mice did not gnaw on the aspen balls at all. The most gnawed items were the aspen chew sticks, which needed frequent replacing. Flat birch sticks were the second most popular gnawing items, making them a good, cost-effective option. Burbidge et al. (2023) also recommend aspen chew sticks after trialing them with CD1 mice, noting that the sticks are easily autoclaved, stored, and added to a cage. However, they too have found that the items need to be replaced frequently (weekly in some cages); moreover, they found that interest waned over time, but was renewed after the sticks were removed for a week. Placing wooden chew sticks on the cage floor may be preferable to providing them between the bars of the lid (King, 2019), although there may be differences between strains in how much they are used and in the animals' preferences for their location.

Several studies have investigated the effects of structural enrichment on group-housed-male aggression. In a study by Gjendal et al. (2017), group-housed male C57BL/6 mice were assessed for aggression, anxiety behavior, and fecal corticosterone metabolites as markers of stress after they were provided a hemp rope hanging from the cage lid. Mice shredded and climbed the ropes, indicating that this may be an engaging addition to the cage. The presence of a single rope had no effect on aggression, stress, or anxiety levels. In a second experiment, they similarly found that the presence of one, two, or seven hemp ropes had no effect on the level of aggression. Notably, in this study, all mice were also provided nesting material, dark acrylic shelters, raised cage lids, cardboard tubes, wooden chew blocks, and treats in the bedding to promote foraging; possibly, if hemp ropes had been provided as the only resource in impoverished environments, they may have caused a spike in territorial behavior. A pronounced increase in aggression *was* seen when mice were frequently tail handled (see chapter on **Human-Animal Interaction**), highlighting the fact that aggression is often a response to stress and not necessarily related to cage contents.

Indeed, Giles et al. (2018) found that the aggression levels of group-housed male BALB/cJ (high aggression) and BALB/cByJ (low aggression) mice did not vary appreciably based on the enrichment item in the cage, whether it was a cotton Nestlet, a shelter, or a suspended mezzanine with an access ramp. But in both substrains, aggression increased after a stressful event (one mouse was removed from the cage, socially isolated for 5 minutes, and then reintroduced), though the increase was smallest in the BALB/cByJ (low aggression) substrain housed with the mezzanine. Lockworth et al.

(2015) documented aggression in male athymic nude mice group housed with one of five enrichment items: crinkle paper, a paper roll, a cotton Nestlet, a nylon bone, or a mouse hut with a running wheel on top. Overall, the type of enrichment had no effect on aggression, although the group given the hut with running wheel experienced an initial spike in aggression in the first week the item was added to the cage. As with other studies, all treatment groups showed a marked increase in aggression after experiencing an external stressor (a cage change and social regrouping).

FEEDING AND FORAGING ENRICHMENT

Hobbiesiefken et al. (2021) provided mice with a variety of foraging puzzles filled with millet seeds, theorizing that this promotes cognitive engagement and increased active behavior. All of the devices—flap puzzle, tube with stones, lattice ball, treat ball, and sliding puzzle—were used by the mice. The most actively used was the flap puzzle, a tent-like device with two reward holes covered by flaps (a 3D printing template is available in the article's supplementary materials). Froberg-Fejko (2010) advocated the use of commercially available pieces of saplings with predrilled holes, which can be packed with different types of food to promote foraging.

ENVIRONMENTAL COMPLEXITY

A common concern among laboratory personnel is that providing group-housed mice with more resources will lead to increased aggression; however, many studies show the opposite effect. In one study, male and female BALB/cJ mice housed with a shelter in addition to other plastic structural objects (e.g., tunnel, hollow cube) demonstrated the lowest levels of aggression, while mice in barren housing experienced the highest levels of aggression and injuries necessitating euthanasia (Swetter et al., 2011). Group-housed male C3H/HeJ mice in shoebox cages with four different resources (Nestlet, one cup of crinkle paper, toilet paper roll, shelter) had significantly less fighting than those with two different resources (Nestlet or crinkle paper, shelter). The authors noted that although it is possible for male mice to form stable groups over time, they had not seen a reduction in aggression over time in their colony until this study (Menke et al., 2018). In another study, group-housed male C57BL/6 mice displayed significantly less aggression when housed in larger cages with bedding, various shelters, running wheels, a tunnel, a wooden gnawing stick, a crawl ball, a tube maze, and nesting material compared to mice housed in shoebox cages with bedding and nesting material only (Aldhshan & Mizuno, 2022). Mice in the more complex condition were observed behaving aggressively approximately 6% of the time, while mice in the shoebox-cage condition were aggressive approximately 32% of the time. Complex housing conditions were also associated with altered expression

of BDNF (a protein that plays a role in controlling aggression and emotions) in various areas of the brain, indicating that the activity of neural-signaling pathways was altered in specific regions of the brain.

Mice in more complex—and thus, more species-appropriate—conditions may exhibit lower aggression because they are generally less stressed (stress is known to exacerbate aggression). Gurfein et al. (2012) assessed fecal corticosterone, body mass, and immune responses in male BALB/c mice group housed under various conditions. Mice were housed in shoebox cages with only bedding (control); shoebox cages with bedding and running wheel (control exercise); larger cages with crinkle paper, hut, and tunnel (enriched); or larger cages with crinkle paper, hut, tunnel, and running wheel (enriched exercise). They found that mice in the control treatment had significantly higher levels of fecal corticosterone and lower body mass compared to all the other groups, even though mice in the control group ate the same amount of food as mice in the enriched group. (Mice in the two exercise groups ate more.) Mice in the control group also had lower spleen mass, and immunological testing suggested that mice in the enriched exercise group had reduced chronic exposure to corticosterone, catecholamines, and other stress-related factors during the 70-day experiment.

Nip et al. (2019) focused on female C57BL/6, BALB/c, and DBA/2 mice housed together (one mouse of each strain per cage), comparing shoebox cages containing nesting material to larger cages containing nesting material and many additional resources (two types of running wheels, two tunnels, two wiffle balls, wooden climbing structure, log bridge, sock hammock, pine cone, suspended egg carton, hanging paper cup, gnawing items, hut). After living in their respective environments for 2 months, mice were observed for stereotypies, social behavior, aggression, and depressive behavior. Averaged across strains, mice in the complex cages displayed 80% less stereotypic behavior and 40% less aggression, even after controlling for their lower rates of social interaction compared to mice in the shoebox cages. Enriched mice were also less depressed: They displayed 50% less inactive-but-awake behavior and 15% lower float times in the forced swim test.* Using the same mice but conducting monthly observations over the lifespan (19–36 months) of the C57BL/6 and DBA/2 strains, Kitchenham et al. (2024) found that the lifetime average rate of stereotypies, which was stable across time, was higher in the shoebox cages; the most common types of stereotypies were bar mouthing and route tracing. Thus, large and complex cages that allowed mice to exhibit a range of behaviors—running,

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* *This methodology is deemed to cause undue harm or suffering in addressing the research question.*

climbing, gnawing, and hiding—reduced aggression, stereotypies, and depression in female mice compared to shoebox cages with nesting material.

Under the same mixed-strain-housing and environmental conditions, Adcock et al. (2021) used a social approach test to determine that mice were able to discriminate between individuals who had been raised under different environmental conditions. In this test, a mouse was released into an arena where she could approach and interact with other mice and novel objects. Mice housed in the shoebox cage conditions were generally less sociable than mice from the more complex conditions; moreover, individuals who were recipients of the most aggression in their home cages sought out mice from the more complex conditions, suggesting that they may have been motivated to seek out less aggressive companions. Similarly, Aujnarain et al. (2018) found increased sociability in a social approach test when mice were housed in more complex conditions. CD1 male and female mice were housed in either shoebox cages (contents not described) or slightly larger cages with metal running wheels and a variety of objects rotated weekly (e.g., plastic balls, Lego bricks, tunnels). Both males and females made use of the running wheels. Mice housed in more complex conditions also demonstrated reduced anxiety in the open field and elevated plus maze tests.

Gygax et al. (2024) investigated behavior and space use in group-housed male and female mice of two strains (C57BL/6 and Swiss) housed in enriched shoebox cages versus large pet-style cages. The shoebox cages contained a mouse house, a tunnel, nesting material, and a gnawing block. The pet-style cages consisted of a base (45 x 22 x 20 in.) made of transparent plastic sheets, topped with a 14-inch-high wire

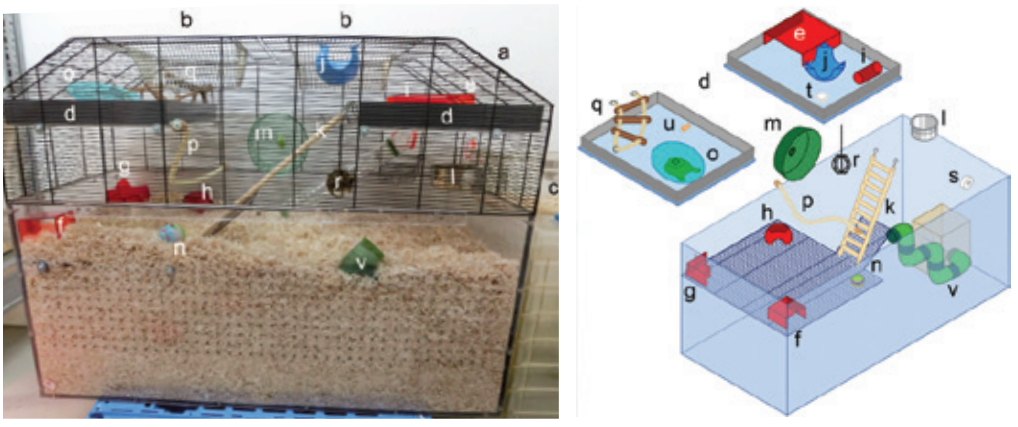

Figure 4: In Gygax et al. (2024), mice housed in these pet-style cages exhibited fewer stereotypies and engaged in more running, locomoting, gnawing, and digging than mice in enriched shoebox cages.

grid (Figure 4). The base was filled with 16 inches of PuraFlake bulk aspen bedding that allowed mice to build burrows that didn't collapse. The cage also contained a multitude of resources, including two shelves, various shelters and tunnels, running wheels, ladders, foraging devices, and gnawing sticks. Mice in the pet-style cages engaged in more running, locomoting, gnawing, and digging. They spent most of the light (inactive) phase in the deep bedding, and most of the dark (active) phase on the disc wheels, shelves, wire grid, or in the deep substrate. Mice in the enriched shoebox cages performed more climbing, rearing, and stereotypic behaviors. The authors concluded that standard housing conditions for mice thwart the animals' natural activity and movement patterns, with inadequate bedding depth and limited opportunities for physical activity likely having the strongest negative impact on the welfare of mice in the laboratory.

Some studies have highlighted how standard housing conditions can negatively impact cognition, with complex environments sometimes reducing these effects. For example, female C57BL/6J mice were housed for 1 month either individually or in groups of two or three, in either barren or complex cage conditions (larger cages containing cotton Nestlets and plastic cage furniture such as tunnels, climbing structures, and igloos). Single-housed mice in barren conditions had lower cognitive performance in a Y-maze test; performance in a novel-object recognition test improved for mice housed with social companions or in more complex cages (Doulames et al., 2014). Similarly, O'Connor et al. (2014) found that mice housed in groups of 3–10 in larger and more complex conditions (rat cages with a variety of objects, including a running wheel) were faster at solving a puzzle task, indicating improved problem-solving abilities, compared to mice housed in groups of 2–5 in smaller cages containing only a red igloo. In another study, male and female C57BL/6 mice were housed in shoebox cages with corncob bedding and a cotton Nestlet, or in larger cages furnished with nesting material, a shelter, and a variety of objects (e.g., pipette tips, test tube racks, bottle caps) rotated weekly. The authors found that mice from the more complex conditions had better performance in a water maze test, indicative of improved cognitive abilities (Hendershott et al., 2016). While the intent of their protocol was to use common laboratory items to enable other labs to easily reproduce the conditions, it is unclear how these items are biologically relevant or beneficial to the animals and raises the possibility that the effects seen were more strongly related to the provision of shelter, nesting material, and increased space than to the other "enrichment" items per se. Using male and female C57BL/6 mice, Dijkhuizen et al. (2024) found that more complex environments (group housing in larger cages furnished with running wheels, walking bridges, climbing rods, tubes and shelters, and wooden sticks) also improves motor performance and motor learning.

Other studies have emphasized the value of novelty by rotating enrichment items regularly. In one example, female C57BL/6J mice were housed in conventional conditions with bedding, nesting material, and a hut, or in more complex conditions with bedding, nesting material, hut, and five items—rotated weekly—from the broad categories of "structural," "housing," "nesting," and "foraging." Mice kept in conventional conditions displayed more stereotypic behavior and inactivity than mice in the complex conditions. Foraging puzzles and running wheels were used actively by mice; a second level (in the form of a shelf, in the "structural" category) was used for both active and inactive behaviors, and increased the amount of available floor space in the cage. Structures affixed to the cage lid were observed in use less than other structures in this study (Hobbiesiefken et al., 2021). Meikle et al. (2020) provided breeding Swiss Webster mice with weekly rotating enrichment objects, consisting of a variety of food items (e.g., mealworms, sucrose tablets, sunflower seeds) and structural items (e.g., PVC tunnels, cardboard tunnels, igloos). There were no effects on reproductive parameters such as litter sizes, but pups from the more complex conditions weighed more at weaning than pups who were housed with only bedding and nesting material (although this could have been impacted by the provision of extra food resources). One study attempted to rank preferences of female C57BL/6J mice for different enrichment items, with the main finding that mice have strong individual preferences (Hobbiesiefken et al., 2023). This highlights the importance of providing varied resources to increase the likelihood that individual needs are being met.

If a structurally complex environment cannot be provided permanently, then there may be some limited benefit to providing it during the sensitive period between birth and weaning. Silva-Almeida et al. (2024) found that male Swiss mice who were housed in a complex environment (larger cage with platform, tunnel, arch, shelter, and paper towels for nesting) from birth until weaning with subsequent housing in barren cages had lower anxiety in the elevated plus maze test compared to mice raised in barren cages from birth. This effect was not observed in females, nor in either sex in other tests of anxiety. Because the benefits of providing environmental complexity only during a short period compared to not providing it at all appear to be limited and may actually result in other negative consequences (see chapter on **Abnormal Behavior**, section on **Rats**, subsection on **Environmental Complexity** below), complex environments should be provided for the duration of the animals' lives.

A better approach to providing mice access to a complex environment when this can't be a permanent feature of their home cage is to provide regular access to playpens. In one study, female mice housed in mixed-strain trios (C57BL/6J, BALB/cJ, and DBA/2J) in shoebox cages with bedding, nesting material, and a shelter were

given access to playpens three times per week for 30 minutes at a time. The playpens were constructed out of two large rat cages, connected by removing a filter from each cage and inserting a tunnel. One rat cage contained burrowing substrate, while the other contained a running wheel, nesting material, and a variety of objects to hide in or climb, such as suspended netting, tunnels, and other climbable structures (Figure 5). Results indicated that mice perceived playpens positively; playpen mice showed increased anticipatory behavior before playpen access while control mice (who remained in shoebox cages) showed no change relative to baseline values. Over time, playpen mice also entered the playpens more quickly, indicating that playpen access was enticing to mice (Figure 6). Mice were active throughout their time in the playpens, demonstrating a range of natural behaviors such as climbing and digging tunnels in the burrowing substrate. Some aggression was seen in the playpens, but this was mainly expressed by C57BL/6 mice and may have been representative of typical home cage dynamics (Ratuski et al., 2021).

In a follow-up article, Ratuski et al. (2024) examined how playpen access affected measures of welfare outside the playpen. They found that following an acute stressor (cage changing), playpen mice of the C57BL/6J strain had lower aggression than control mice; aggression in the other two strains was low throughout the study. Playpen mice also scored lower on some measures of anxiety in the elevated plus maze tests and the open field test. However, playpen mice had higher rates of stereotypies in

Figure 5: In Ratuski et al. (2021; 2024), playpens for mice consisted of two interconnected rat cages. One cage (left) contained climbable structures, and the other (right) contained burrowing substrate.

Figure 6: Mice entered the playpen voluntarily using a tunnel placed in their cage (Ratuski et al., 2021; 2024).

their home cages compared to control mice. This result indicates that playpen access at the frequency and duration provided in this study was not enough to mitigate the development of stereotypies; indeed, it made them worse. The authors theorized that higher rates of stereotypies in playpen mice may indicate one of three things: (1) a more negative affective state (mice who know about the existence of more complex environments perceive the home cage more negatively); (2) increased motivation to escape the home cage (bar mouthing, in particular, is thought to develop from such a motivation); or (3) in accordance with the "coping hypothesis" of stereotypic behavior, increased agency in the playpen contributing to more active coping mechanisms.

AUDITORY ENRICHMENT

Alworth and Buerkle (2013) reviewed a number of studies that assessed the effects of music on measures of physiology and behavior in rodents and other species. For example, weanling mice exposed to Mozart's music had better learning ability than mice exposed to Beethoven's music. In another study, mice exposed to "serenade" music had lower stress levels (lower ACTH and noradrenaline) than mice exposed to "march" music or no music. One study found that mice who were exposed to a fire

alarm overnight* had lower immune function (lower thymus weight, T-cell proliferation, and NK cell activity) and higher stress levels (higher ACTH) compared to nonexposed mice; those who were subsequently exposed to Herbert Von Karajan's *Adagio* album had lower stress levels (lower ACTH) than those who were exposed to the alarm alone.

Li et al. (2010) exposed male mice with knock-in BDNF genotypes (expressing an anxious phenotype) to white noise or a selection of music (classical Chinese, classical Western, children's choir) daily for 6 hours. Mice exposed to music had higher BDNF expression and lower anxiety in the elevated plus maze test and open field tests compared to mice exposed to white noise.

Rats

NESTING MATERIAL

Contrary to widely held beliefs, rats will build nests when suitable resources are available or when they are highly motivated, such as when they are cold, pregnant, or nursing. In nature, Norway rats typically use organic material such as leaves and grass to line their nesting chamber. In the laboratory, rats may prefer to rest inside shredded paper nests rather than typical open-ended PVC tunnels (Bradshaw & Poling, 1991; Patterson-Kane et al., 2001).

Vitalo et al. (2012) demonstrated therapeutic effects of nesting material for single-housed Sprague Dawley rats used in wound-healing research. Rats housed singly with cotton Nestlets or wood pulp nesting material had significantly better wound healing than rats without nesting material, although rats with cotton Nestlets had a slightly faster rate of healing than rats with wood pulp material. To test the effect of nesting material on self-injury related to overgrooming, Khoo et al. (2020) housed male Long-Evans rats in shoebox cages containing a nylon bone and a shelter, and either with or without crinkle paper (Enviro-Dri). Although there was no difference in the overall prevalence of overgrooming-related self-injury (e.g., hair loss and skin inflammation or lesions), rats with nesting material had lower severity of these injuries. Comparing the effects of sex, vendor, facility, and prior experience with nesting material on rat nest quality, Schwabe et al. (2020) found no consistent patterns and high day-to-day variation. However, in a pilot study leading up to this work, they identified that for pair-housed rats, 28 grams of crinkle paper was more suitable than 14 or 21 grams.

** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

Some scientists investigate the impacts of environmental enrichment on various translational disease models. To investigate the potential therapeutic effects of environmental enrichment on a rat model of adverse childhood experiences, Corredor et al. (2022) exposed male and female Wistar rats to an early life stress protocol. They found that these rats had better performance in the Morris water maze test if they were provided with sealed teabags filled with different nesting materials (bedding material, cardboard pieces, or rope segments) compared to those without nesting materials. Stressed females with nesting materials also demonstrated lower anxiety in the elevated plus maze test.

HUTS, TUNNELS, AND NEST BOXES

Rats are often provided with open-ended PVC tunnels (or "tubes") as shelter, but there is evidence that rats prefer other sheltering resources over open-ended tunnels, such as nesting material and larger nest boxes with one open end (Bradshaw & Poling, 1991; Patterson-Kane, 2003; Patterson-Kane et al., 2001).

Commercially available tunnels may be too small for adult rats, or may only allow one rat to shelter at a time (Figure 7). Newman (2010) obtained larger plastic tunnels from a local plastic retail company (inner diameter: 4 in.; length: 8 in.) and used a hot glue gun to glue 3–4 tunnels together, with the third and fourth tunnels stacked on top of the first two. The stacked structure occupied less floor space than the same number of single tunnels placed next to each other and allowed rats to rest on top and

Figure 7: Standard PVC tubes are too small to fit two adult rats. Larger, tent-like structures are more suitable, as they permit rats to cuddle together.

Figure 8: In Collier (2010), a red-tinted glass tube with one open end and one sealed end was the shelter design most preferred by rats in metabolic caging.

observe their surroundings from a higher vantage point. When provided with recycled glass jars and plastic shelters in another facility, rats made use of both plastic and glass shelters, but tended to prefer glass (Rossi, 2017).

As described above in the section on mice, Collier (2010) designed red-tinted glass shelters for use in rodent metabolic cages. The most popular shelter for rats was a red-tinted glass tube with one open end and one sealed end (Figure 8). The tube was fixed to the floor of the cage and had small holes for ventilation; rats slept and groomed inside the tube and climbed on top of it to sit and rest.

Rats with exteriorized devices such as head caps or telemetry are sometimes deprived of environmental enrichment on the grounds that they may become entangled or entrapped. Hawkins (2014) noted that simply not providing enrichment to these animals is not an appropriate solution; instead, enrichment should be adapted to these animals' unique needs. For example, rats with exteriorized devices can be provided with nest boxes that have a wide entrance, are as tall as the cage to prevent rats from climbing on top, and are fixed in place to ensure the animals (or the instruments) are not caught between the shelter and the cage wall (Figure 9).

Using rats as a model of early childhood adversity, Corredor et al. (2022) found that providing a variety of PVC tunnels improved cognitive responses in the Morris water maze in male and female Wistar rats exposed to early life stressors, although results varied depending on the type of stress to which they had been exposed.

STRUCTURAL ITEMS

Rats can benefit from voluntary wheel running through increased exercise, improved cognition, and more positive emotional states. In a pilot study, female Wistar rats made use of plastic running plates for sitting, standing, and running, although individuals varied in how long they ran each day (Frei et al., 2021). Heyse et al. (2015) recorded ultrasonic vocalizations in male Wistar rats with access to an upright running wheel that was either locked in place or unlocked and free to rotate. Rats who engaged in running emitted 50-kHz calls, indicative of positive emotional states, both before and during wheel access. Rats with access to an unlocked running wheel also approached the wheel more quickly over time. Li et al. (2021) found that voluntary wheel running improved cognitive functioning of male Wistar-Kyoto rats, as demonstrated through improved radial arm maze performance and electroencephalogram (EEG) results. When comparing the effects of voluntary wheel running on single-housed versus group-housed rats, Lynch et al. (2019) found that rats housed in groups ran significantly greater distances than single-housed rats, indicating that wheel running may be a socially modulated activity. Single-housed rats with access to running wheels demonstrated lower anxiety in the elevated plus maze test compared to rats without wheel access. When wheel access was discontinued, rats who previously had access to running wheels had greater intake and preference for ethanol (indicative of anxiety) compared to rats who had never been given wheel access. This study highlights the negative consequences of removing access to valued resources, which will be discussed further in the subsection on Environmental Complexity.

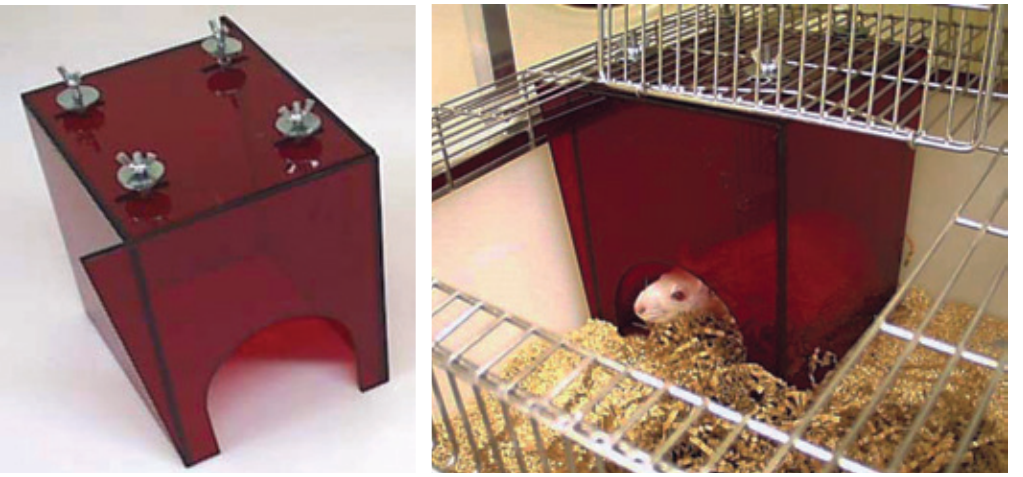

Figure 9: Instrumented rats can be provided with tall nest boxes that have a wide entrance and are fixed in place (Hawkins, 2014).

Gnawing items can be safely used with rats. Aspen wood gnawing blocks did not adversely affect a wide range of physiological outcomes typically used in exploratory toxicology studies when tested with male Sprague Dawley rats (Ditewig et al., 2014). There were some effects on body weight, but subsequent studies with larger sample sizes failed to replicate this result. Typically, gnawing devices are discarded within 4 weeks or less because of concerns for animal health. To assess whether these concerns are warranted, Danner and Rao (2017) assessed the cleanliness and health impacts of nylon chewing bones left in male Sprague Dawley rat cages for 4, 8, or 12 weeks. Bacterial load, debris accumulation, and various blood markers were not affected by prolonged use of unsanitized gnawing chews. Although there was an initial increase in bacterial loads and debris accumulation at week 4, these subsequently dropped by weeks 8 and 12, leading the authors to conclude that

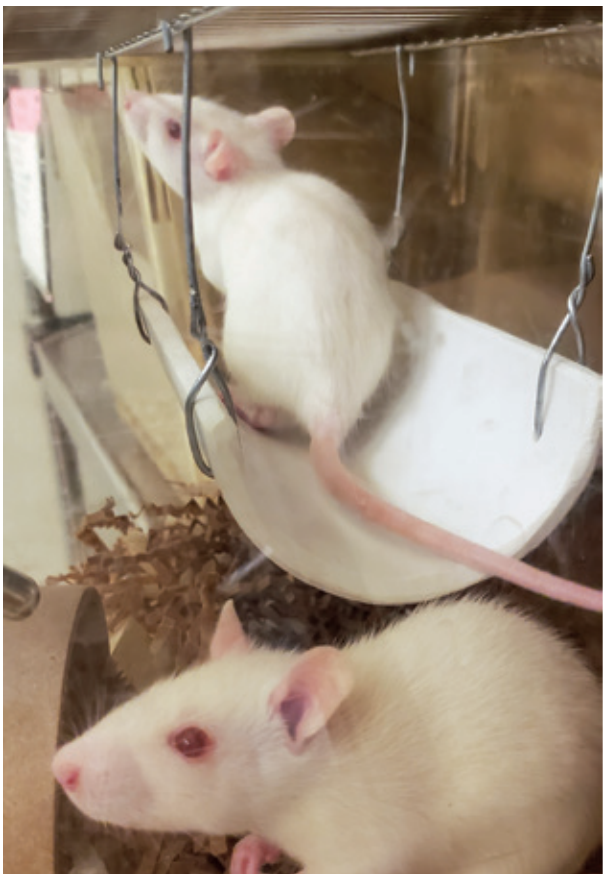

Figure 10: Brekke and Scholz (2020) showed that durable and cost-effective plastic hammocks for rats can be crafted out of PVC pipes cut in half lengthwise and hung from the cage lid.

gnawing devices left for longer periods of time do not pose risks to rat health. Strickland (2023) found that single-housed Sprague Dawley rats made more use of destructible enrichment made of cardboard versus paper, and of items stuffed with hay versus shredded paper. The most successful DIY design consisted of an autoclaved toilet paper roll stuffed with timothy hay, with both ends folded over like a box, and four slits cut into the top and bottom of this "cardboard gift box."

Laboratory research and observations of pet rats suggest that these animals enjoy elevated platforms such as hammocks, shelves, or suspended lofts (e.g., Neville et al., 2022; Patterson-Kane et al., 2001). As a DIY option, Brekke and Scholz (2020) crafted plastic hammocks for rats out of PVC pipes cut in half lengthwise and hung from the cage lid using 16-gauge wires that were sanded to remove sharp edges (Figure 10). These were durable, easily sanitized, and cost effective.

FEEDING AND FORAGING ENRICHMENT

Blackburn et al. (2020) recommended providing a commercially available forage mix in the bedding of the home cage; the mix contains sunflower seeds, dried banana, maize flakes, and locust beans, which rats find appetizing. Organic wheatgrass can also be grown aeroponically (i.e., without soil, greatly reducing risk of fungal or bacterial growth) and provided as environmental enrichment to rabbits and rodents to promote foraging. Brown (2010) recommends placing the root mat outside the wire lid of the cage with the grass blades extending into the cage or hiding other treats within the blades of the grass to promote foraging.

In a 2-year experiment, Laaksonen et al. (2017) assessed the welfare impacts of a wooden diet board used to feed male and female Sprague Dawley rats. The board contained grooves where food pellets could be tightly packed, and the rats were required to gnaw at the wood to release the pellets. Compared to rats who were fed from the food hopper, rats fed via a diet board spent significantly more time eating and less time grooming in 24-hour cycles, and males spent less time resting. Female rats with diet boards had higher fecal corticosterone metabolites than food-hopper-fed female rats, while there was no difference between the two in males; this result may reflect increased physical activity rather than stress. There was no effect on anxiety in the elevated plus maze test.

ENVIRONMENTAL COMPLEXITY

Valuable information about appropriate environments for rats can be gleaned by looking at what is considered normal for pet rats, whose living environment is less

likely to be constrained by space and economics. A team of veterinary scientists developed guidelines for pet rat housing based on consultations with experts, half of whom had worked in an animal research facility or a veterinary department at a university (Neville, Hunter, et al., 2022). The guidelines comprise several key factors considered to be "important and necessary" for good housing. These include a complex environment with multiple levels, digging opportunities, opportunities to exercise, refuge areas, and suitable nesting substrates. Furthermore, a survey of pet rat owners in the United Kingdom revealed that the median number of enrichment types provided to pet rats was seven. The most common items provided (each of which, individually, appeared in at least three quarters of the responses) were a suspended area, a climbing structure, a shelter, enrichment activities (e.g., bobbing for peas), digging substrate, a tunnel, a foraging device, and nesting substrate (Neville, Mounty, et al., 2022). Rats provided with more types of enrichment were more likely to engage in climbing, digging, nesting, and "boggling" (which consists of the rat's eyes "popping" in and out and is indicative of a content emotional state). A similar survey of German-speaking pet rat owners found that the majority of rats received a variety of enrichment objects, with nearly all rats having access to shelters, nesting material, hammocks, and tunnels (Schneidewind et al., 2024).

A series of experiments led by Abou-Ismail demonstrated the positive effects of more complex housing for rats. In one study, male Wistar rats were group housed in barren cages or with a variety of environmental resources (shelter, nesting material, aspen wood block, perch, and weekly rotating items such as nylon bone, rope, or ladder). Rats with environmental resources showed longer bouts of sleep (poor or fragmented sleep is an indicator of anxiety and depression) and less aggressive behavior (Abou-Ismail et al., 2010). In a similar experiment, male Wistar Hannover rats were single housed in barren cages or with a variety of resources (shelter, nylon bone, crawl ball, ladder, and cotton Nestlet). Rats provided with resources displayed higher levels of sleep, activity, and exploration, while rats in barren cages spent more time stationary or engaging in bedding-directed activity. Rats in barren cages also showed higher levels of anxiety in the elevated plus maze test (Abou-Ismail & Mahboub, 2011). In another study, male Wistar rats were group housed with one environmental resource (either shelter, aspen wood block, nylon bone, ladder, or crawl ball) or with all of the available resources; rats with all the resources demonstrated improved measures of good welfare, such as more sleeping, less inactive-but-awake behavior (less of this behavior suggests lower levels of boredom), less aggression, and higher spleen and thymus weights (which indicates lower stress levels; Abou-Ismail, 2011). A follow-up study investigated the importance of novelty versus complexity. Male Wistar rats were group housed for 5 weeks with either five copies of the same object, which

was changed every week (novelty condition) or five different objects in the cage throughout the study period (complexity condition). Rats in the complexity condition had more indicators of positive welfare and fewer indicators of negative welfare; the study outcomes were similar to those tested in the lead author's previous work (e.g., sleep, aggression, inactive-but-awake behavior, organ weights, etc.; Abou-Ismail & Mendl, 2016). Finally, in a study investigating the relative importance of social housing versus enrichment, male Wistar rats were housed in shoebox cages with elevated lids in one of three conditions: single housed, group housed, or single housed with a variety of resources (shelter, nylon bone, ladder, and crawl ball). Rats housed with environmental resources explored more during the dark phase, slept more during the light phase, and had higher spleen and thymus weights. One of the important implications of this study is that social housing alone does not compensate for an inadequate physical environment (Abou-Ismail et al., 2014).

A similar study from another group replicated the general findings from the series of studies led by Abou-Ismail. Male Wistar rats were group housed in one of three conditions: barren cages, cages containing one of three items (cardboard tube, plastic cylinder, or disposable cap) rotated weekly for novelty, or cages containing three of five items (disposable cap, ping-pong ball, acrylic tube, acrylic cabin, acrylic hut) rotated weekly for novelty. Rats housed with one or three items demonstrated lower anxiety in the elevated plus maze test compared to rats without any items. Rats housed with three items also demonstrated reduced responses to acute and chronic painful stimuli (Kimura et al., 2019).

Focusing on the emotional benefits associated with housing in complex environments, Brydges et al. (2011) demonstrated that male Sprague Dawley rats housed in barren shoebox cages had pessimistic responses in a cognitive bias test; when they were moved into larger, more complex cages containing a deep layer of wood shavings, two cardboard tubes, two cardboard houses, four wooden blocks, and one plastic hut, they demonstrated significantly more optimistic responses, indicating improved mood. In another study assessing rat emotional states, female Sprague Dawley rats were housed either in standard shoebox cages containing a PVC pipe and paper towels as nesting material or in large, semi-naturalistic wire cages with four levels connected by ramps. In these latter cages, the bottom level was filled with soil to a depth of 1 foot for burrowing, and other levels contained litter boxes, PVC pipes, a hammock, a lava rock, horizontal ropes, and (on occasion) shredded paper or timothy hay (Figure 11). Rats housed in standard cages demonstrated more hyperactivity in anticipation of a treat, indicative of greater reward sensitivity and therefore a more negative emotional state (Makowska & Weary, 2016).

Figure 11: A semi-naturalistic cage for rats with multiple shelters, climbable structures, and soil for burrowing (Makowska & Weary, 2016).

Despite its ability to reduce stress in some contexts, increased environmental complexity may not negate the physiological impacts of acutely stressful experiences. For example, female Sprague Dawley and spontaneously hypertensive rats were single housed under one of two conditions: a barren cage containing

only bedding or the same type of cage supplemented with a shelter, a gnawing toy stuffed with paper and treats, and a shreddable pack of corncob and wood chips. Spontaneously hypertensive rats were more active in the comparatively complex condition (an effect not seen in the Sprague Dawley rats). In neither strain, however, did environmental complexity result in decreased heart rate or blood pressure in response to stressful procedures (Azar et al., 2012). In a similar experiment, male and female Sprague Dawley and Wistar rats were single housed in a barren cage or in a more complex cage with three social partners and access to nesting material, foraging items, and a place to hide. When the rats in both environments remained undisturbed, activity was greater and heart rate lower, generally, for those in the more complex environment. When the rats were subjected to stressful procedures, however, those in the more complex environment did not experience a comparative reduction in heart rate or blood pressure (Sharp et al., 2014).

Increased environmental complexity may be beneficial for brain development. Pair-housed male Long-Evans rats housed in enriched cages (large cages with new sets of objects every few days, including a running wheel that was locked to control for exercise) had better performance in a water maze (indicative of better cognition and memory) and lower oxidative stress in the hippocampus (oxidation is a neurodegenerative process) than pair-housed rats in smaller, barren cages (Mármol et al., 2017). In another study, adolescent Sprague Dawley rats were housed in either a "physical enrichment" condition (two rats with climbing structures, running wheels, tunnels, bedding, and rotating novel toys), a "social enrichment" condition (six rats in a larger cage), a "social and physical enrichment" condition (six rats in larger cage with the aforementioned enrichment items), or a "standard" cage (two rats without the enrichment items). Housing conditions significantly affected neurological outcomes, such as increased spine density in animals housed with more social partners and environmental resources (Gabriel et al., 2020). Exposure to enrichment (larger cages containing a hut, a running wheel, three levels connected by ramps or tunnels, and toys rotated weekly) also reduced drug-seeking behavior for single-housed male Sprague Dawley rats previously exposed to heroin, methamphetamine, and nicotine; this reduction in drug-seeking behavior is believed to relate to environmental enrichment's impact on general neurobiological mechanisms (Sikora et al., 2018).

Complex permanent housing is recommended for rats. If this is not possible, however, rats can still benefit from temporary but regular access to a complex environment such as a playpen. For example, King (2019) highlighted the use of playpens made of old, modified rabbit cages filled with bedding, tunnels, water trays, nesting material, and cardboard glove boxes as an effective enrichment strategy, and Blackburn et

al. (2020) found that regular access to playpens containing shelters, plastic balls, cardboard glove boxes, and cardboard tunnels for rats used in cancer imaging studies helped the animals form a positive association with leaving the home cage. Similarly, Gudbrandsen (2024) found that male Zucker diabetic Sprague Dawley rats who were pair housed in enriched, individually ventilated cages stopped exhibiting stereotypic leaping in one spot, rocking, and licking/biting their home cage within 10 days after the facility implemented regular visits to a playpen following handling and experimental procedures. The author noted that the rats quickly learned that handling and experimental procedures were "rewarded" with playtime and became more cooperative and easier to work with. Hinchcliffe et al. (2022) examined the influence of playpens on the affective states of male Lister hooded rats. The animals were pair housed in well-resourced conventional cages (woodchip and paper bedding, cotton rope, gnawing wood block, cardboard tunnel, and large red house) and were tested during their active phase under red lights. They were placed in one of three arenas, each measuring 40 x 40 x 24 inches: a "playpen" containing a variety of objects, including platforms, ladders, fern cones, tennis and dryer balls, tunnels, and shelters; a "ball pit" filled with 400 plastic balls; or a "control" arena, which was empty. During one 5-minute stay in the playpen or the ball pit, rats emitted about tenfold more 50-kHz vocalizations, indicative of a positive affective state, compared to the empty arena.

A number of studies have investigated the therapeutic effect of playpens on translational disease models. In addition to their test of rats' affective state while in a playpen or ball pit described above, Hinchcliffe et al. (2022) investigated whether a playpen or ball pit could mitigate symptoms in a rat model of anxiety and depression. They found that rats who were placed in a playpen or ball pit for an hour not long after they were injected with an anxiogenic and pro-depressant drug had a significantly less negative affective state in an affective bias test compared to rats who were not placed in the play areas after injection. To investigate the potentially therapeutic effects of playpens on chronic and acute stress, Scarola et al. (2019) pair housed male Long-Evans rats in barren cages and divided into five treatment groups: an unmanipulated control group, a chronic stress group (exposed to 30 minutes of predator sounds, 5 days per week for 6 weeks) with playpen access, a chronic stress group without playpen access, an acute stress group (exposed to 30 minutes of predator sounds, 1 day per week for 6 weeks) with playpen access, and an acute stress group without playpen access. The playpen consisted of an open field arena filled with a variety of objects that rats could climb, hide within, or play with; playpen access was given for 30 minutes, 3 days per week for 6 weeks. Rats in the chronic and acute stress groups with playpen access had lower corticosterone, testosterone, and IL6 (a pro-inflammatory cytokine) and higher oxytocin (a hormone associated with positive affiliation), DHEA (a

hormone that helps reduce stress), and IL10 (an anti-inflammatory cytokine) than rats who experienced the same stressors without playpen access. Rats exposed to acute stress with playpen access had the healthiest profile in terms of stress regulation and immune activation, suggesting that a little stress and exposure to enrichment is healthier than living in a monotone, barren environment. Another study corroborated the results that playpens have a therapeutic effect on a rat model of chronic stress. Male Sprague Dawley rats were single housed in barren cages and divided into four treatment groups: an unmanipulated control group, an "EE" group with playpen access 2 hours per day, 5 days per week, for 7 weeks, a "CMS" group exposed to chronic, mild, unpredictable stress 7 days per week for 3 weeks, and a CMS+EE group that experienced the stressor but also had playpen access. The playpen consisted of a large cage with three floors, ramps, wheels, and objects that were rotated weekly; rats were placed in the playpen in groups of six. Playpen access reduced anxiety in the elevated plus maze test and resulted in lower secretions of norepinephrine for rats in both the chronically stressed (CMS+EE) and unstressed (EE) groups. Additionally, stressed rats with playpen access (CMS+EE) showed lowered hormonal responses to the stress and reduced behaviors indicative of anxiety, depression, or memory impairment compared to their CMS only counterparts (Costa et al., 2021).

Monnas (2021) provided tips for successful implementation of a playpen. Rats from their facility's training colony had access to a cage designed for finches (9-cage

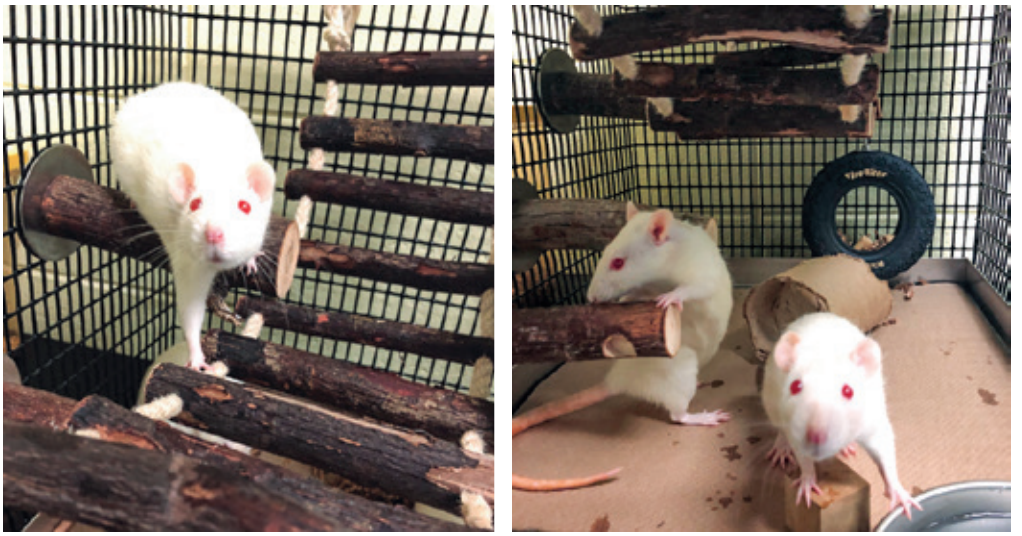

Figure 12: In Monnas (2021), ascending wooden perches leading to a hanging bridge encouraged climbing and vertical stretching in this playpen for rats.

finch housing unit) containing a variety of structures such as hammocks, perches, nesting material, tunnels, and gnawing blocks (Figure 12). They noticed that rats were timid and hiding when placed in the cage by themselves but showed "an exponential increase in curiosity" when placed there with another rat. Wooden perches placed in an escalating pattern and leading to a hanging bridge encouraged climbing and vertical stretching. When it was time to return rats to their home cage, opening the playpen and allowing rats to climb out on their own (onto the counter from which they could be gently picked up) worked best. Notably, not only did the rats seem to benefit, but facility staff also appreciated the playpen, as it relieved workplace stress and helped to acclimatize these rats to human handling. Gudbrandsen (2024) introduced rat pairs to a playpen gradually: The first two sessions were 5 minutes each; thereafter, stays in a playpen were extended by 5 minutes every second session up to a maximum 20 minutes. Rats were very tentative in the playpen at first, waddling rather than walking and showing no interest in using the platforms, climbing the ladders and suspension bridge, or interacting with the toys (plastic ball, roller with bell). However, after a few sessions, they were running up and down the ladders, engaging in rough-and-tumble play, popcorning (a sign of joy), and moving the toys and huts across the floor and between platforms.

If temporary enrichment such as playpens are used, it is likely important for these to be provided on a regular and ongoing basis, as there is evidence that loss of enrichment can negatively impact welfare. For example, Morano et al. (2019) gave female Sprague Dawley rats nightly access to a larger cage with additional social companions, crinkle paper, metal ladders, plastic huts, and a rotation of novel objects such as nylon bones, cotton Nestlets, and various plastic objects. Some of the rats then had their access discontinued (and remained in barren shoebox cages); control groups remained in shoebox cages throughout the experiment (either single or pair housed). Enrichment removal induced negative emotional states: Rats who lost access to the playpen showed decreased sucrose consumption in a sucrose preference test and increased rates of weight gain relative to control animals who never had access to a playpen. The negative consequences of enrichment removal have also been found with male Sprague Dawley rats: Smith et al. (2017) demonstrated that moving rats from social housing in large cages with a rotating variety of resources (e.g., tubes, balls, ladders, nesting material) into single housing in shoebox cages produced behavioral and physiological outcomes consistent with depression, such as helplessness, overeating, and hormonal responses to stress. Indeed, they concluded that the removal of long-standing enrichment can be used as a model to study the neurobiological effects of loss and the development of psychiatric disorders.

AUDITORY ENRICHMENT

In a review of studies that investigated the impact of music on the welfare of various species, Alworth and Buerkle (2013) described a variety of positive effects on rats. For example, rats exposed to "comfortable" music in utero had better neurogenesis in the hippocampus and better spatial learning ability compared to rats not exposed to music, while rats exposed to noise in utero had the opposite effects. Similarly, rats who had been exposed to music by Mozart in utero and soon after birth had better learning ability than rats who had been exposed to silence, white noise, or music by Philip Glass. Rats injected with tumor cells and exposed to Herbert Von Karajan's *Adagio* album with or without prior exposure to a fire alarm overnight* had fewer metastatic lung nodules than those exposed to only the alarm or nothing at all. Rats in one study preferred music by Mozart over Schoenberg, but only if they had previously been exposed to Mozart's music.

In a review of 11 studies examining the effects of auditory enrichment on rats, Snowdon (2021) observed that eight of the studies compared Mozart (K. 488 in four cases) to something else (usually noise and/or silence) and generally found that the Mozart music had positive effects on anxiety, blood pressure, and learning.

Akiyama and Sutoo (2011) investigated the effects of different music frequencies on rat welfare. They played Mozart's K. 205 on repeat for 10 hours to spontaneously hypertensive male rats, but filtered out various frequencies. They found that rats appeared calmer and their systolic blood pressure decreased the most when exposed to the full-frequency (normal) and high-frequency (4,000–16,000 Hz) versions, decreased moderately in response to the mid-frequency (250–2,000 Hz) version, but remained the same in the low-frequency (32–125 Hz) version or when not exposed to music. These effects were seen for the first 6 hours of exposure to the full-, high-, and mid-frequency music; after 6 hours, blood pressure began to rise back to pretest levels.

In Krohn et al. (2011), male rats (HsdOla:LH and NTac:SD strains) presented with pairings of 60 dB auditory stimuli could choose which they preferred to listen to: silence, white noise, pop music, people speaking, or radio. Silence was the overall top choice, with Sprague Dawley rats preferring silence over any other stimulus, and Lister hooded rats preferring silence over pop music or radio. Neither strain showed a preference for white noise, and both strains preferred the sounds of speaking over radio.

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* *This methodology is deemed to cause undue harm or suffering in addressing the research question.*

Other Rodents

NESTING MATERIAL

In the absence of nesting material, **Syrian hamsters** prefer pine shavings as bedding substrate because the shavings can also be used for nesting (a behavior they are highly motivated to perform); if paper nesting material is provided, other types of bedding substrates may be suitable (Lanteigne & Reebs, 2006). A recent survey of pet Syrian hamster owners revealed that hamsters provided with deeper bedding were less likely to exhibit bar mouthing; at least 8 inches appeared to be best (Fox & Neville, 2024).

HUTS, TUNNELS, AND NEST BOXES

Breeding pairs of adult **Mongolian gerbils** displaying stereotypic digging and jumping were provided with opaque nest boxes inside their home cages. To simulate a burrow-like entrance to the nest box, researchers attached one of four different entry tunnels: straight, L-shaped, T-shaped, or Y-shaped. None of these entry tunnel shapes alleviated stereotypic digging or jumping (Habenicht et al., 2022). Earlier studies, however, found that when a downward-sloping tunnel leading to an opaque nest box was attached below a gerbil cage (with the tunnel accessible through a hole in the cage floor), stereotypic digging was almost entirely prevented in young animals (Wiedenmayer, 1997) and significantly reduced in adults (Waiblinger & König, 2004). It is possible that to be effective in preventing stereotypic digging in gerbils, the nest box must be "below ground."

In a study on shelters for single-housed male and female **Syrian hamsters**, animals were housed in a double-cage setup, where each cage contained food and water, a running wheel, and pine shavings for bedding and nesting, but only one cage contained one of four types of opaque shelter: a 3-inch-diameter, open-ended tunnel either 6 inches or 4 inches in length or a box open on one side and either 6 x 2.5 x 3 inches or 4 x 3 x 3 inches in volume. Hamsters showed no preferences for nesting (i.e., sleeping) in cages with or without a shelter, except when the shelter was the 6-inch tunnel, in which case they spent about 75% of their time in that cage—whether or not they actually used the tunnel for nesting. (When not nesting in a shelter, hamsters nested under the wheel 95% of the time; nesting was defined as a mound of bedding pushed up around the hamster.) When the shelter was a 6-inch tunnel, 21 of 30 hamsters nested more than 50% of the time inside it, whereas only two or three animals nested consistently inside any of the other three options. In a follow-up experiment, a greater variety of shelters were tested in pair-wise comparisons. Of

all the options tested, both male and female hamsters preferred to nest in a 6-inch tunnel that was closed on one end rather than open ended (Veillette & Reebs, 2011).

FEEDING AND FORAGING ENRICHMENT

As an inexpensive foraging enrichment option for **guinea pigs**, Buchanan (2020) recommended cutting cardboard into interlocking circles that can be stuffed with hay or other approved treats (Figure 13).

Like other rodents, **hamsters** need to gnaw to wear down their continually growing teeth. Watson (2020) determined that medium-sized aspen bricks were longer lasting and less prone to surface damage and soiling than aspen balls and large aspen bricks,

Figure 13: To create foraging enrichment for guinea pigs or rabbits, autoclaved recycled cardboard can be cut into six interlocking circles and stuffed with hay or other treats (Buchanan, 2020).

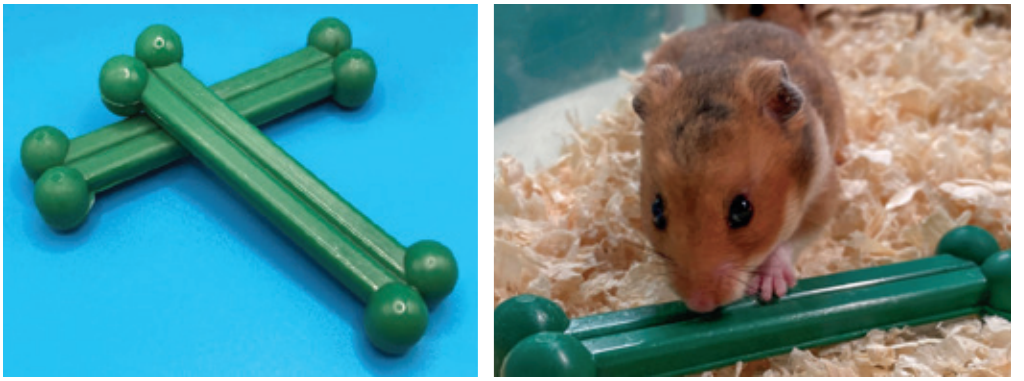

Figure 14: In Devine and Boratyn (2024), nylon green bones were found to be a safe and durable gnawing alternative to wooden chew items for hamsters.

although aspen balls were most engaging for the **Syrian hamsters**, as they were chewed more frequently and could be more easily carried or manipulated. Devine and Boratyn (2024), however, found that medium-sized wooden chew blocks designed for rats splintered in Syrian hamsters' chew pouches and caused noncancerous pyogranulomas. Aspen balls brought in as an alternative to the blocks also caused pyogranulomas, in addition to stretched muscles in hamsters' cheek pouches and oral cavity lesions. Oral lesions caused weight loss and multiple masses and were the cause of death for 6% of the animals. Nylon green bones were found to be safe and durable gnawing alternatives that don't splinter (Figure 14). Hamsters did not ingest these bones despite repeated gnawing; moreover, no hamsters developed pyogranulomas, and the incidence of oral lesions causing death decreased to 0.2% of the animals.

ENVIRONMENTAL COMPLEXITY

Using pair-housed female **guinea pigs** (IAF hairless and Hartley strains), Brewer et al. (2014) placed approximately 10 animals, for 1 hour daily for 1 month, in a fleece-lined open arena that provided increased space and opportunities for social interactions as well as access to wooden gnawing blocks and western timothy hay. Animals from both strains frequently engaged in locomotion, social interactions, resting, and consumption of hay while in the arenas. Hartley guinea pigs demonstrated more social behaviors than hairless guinea pigs. The animals did not display any aggressive biting, while other antagonistic behaviors, such as kicking or chasing, were rare. There were also no effects on salivary cortisol after the animals visited the arena.

Environmental complexity can improve emotional states in **hamsters**. Group-housed male **Syrian hamsters** housed with a running wheel and two spirally wound cardboard

tubes received additional resources (deeper bedding, nesting material, two colored transparent plastic huts, suspended tent, four hamster gnaw sticks, and wooden ledge). After a week, the extra resources were removed from some cages while even more resources were added to other cages. The authors found that hamsters were more likely to approach an empty drinker at ambiguous locations (i.e., they were more optimistic about the likelihood of receiving a reward from an uncertain location, indicative of positive shift in emotions) when resources had been added to their cage for the previous week, compared to hamsters whose resources had been removed. There were no differences between treatments in exploratory behavior, anxiety, or fear of novelty in other behavioral tests conducted (Bethell & Koyama, 2015).

Grippo et al. (2014) investigated the potential therapeutic effects of environmental enrichment on a **prairie vole** model of affective disorders arising from social isolation. Voles were either single or pair housed in a barren cage or in a cage containing several resources (running wheel, wooden block, bowl of food, pellets, igloo, nesting material, several plastic toys, and other miscellaneous objects). Voles housed singly in barren cages were less mobile in the forced swim test* and less exploratory in the open field and elevated plus maze tests, indicating greater depressive behavior and anxiety than voles housed with a social partner or with greater environmental complexity. In a second experiment, all voles were single housed under one of three conditions: barren cage, enriched cage as described above, or barren cage with a running wheel for exercise. After 4 weeks of social isolation, voles in the barren cages displayed the highest levels of depressive behavior and anxiety compared to voles given access to a running wheel or greater environmental complexity. For some outcomes, the running wheel was as effective as the more complex environment; for other outcomes it was intermediately effective. In both experiments, voles housed alone and without access to any environmental resources were most likely to experience negative affective states related to anxiety or depression.

Kapusta et al. (2022, 2023) assessed the short-term reactions of **bank voles** and **common voles** to the addition or removal of environmental resources (aspen gnawing brick and wooden tunnel), respectively. Bank voles reduced stereotypic behavior when the items were added, and they spent more time by the tunnel than by the brick. When the items were removed, stereotypies increased. Common voles, on the other hand, performed more stereotypies when the items were added and did not

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** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

Figure 15: Chinchillas should be provided with running wheels, wooden blocks for chewing, and vertical spaces enhanced with perches and flat-topped huts to allow them to jump, climb, perch, and hide.

significantly change their behavior after removal. However, because the impact of adding or removing these items was assessed for only 30 minutes, the animals may have been demonstrating an initial reaction to novelty (a change in their environment) rather than a lasting behavioral impact.

Chinchillas evolved in rocky mountainous regions and are therefore adept at jumping, climbing, and perching. To provide laboratory-housed chinchillas with opportunities to express some of these natural behaviors, LaFleur and Williams-Fritze (2020) advocated the use of running wheels and vertical spaces enhanced with perches and flat-topped structures (Figure 15). Like mice and rats, chinchillas are burrowing rodents who benefit greatly from having a hut they can retreat into for safety. They should also have gnawing objects such as manzanita sticks or wooden chew blocks. Describing successful improvements at their facility, Mantz and Pugerud (2024) advocated frequent provision of dust baths (see chapter on **Colony Management**), as well as housing them in groups within large cages that allow for perching and climbing and are furnished with running wheels and cardboard foraging boxes stuffed with hay and chew blocks. Implementation of these well-resourced cages has led to increased social interactions and activity levels in the chinchillas. Studying farmed chinchillas, Łapiński et al. (2023) found that single-housed females living in larger cages with two suspended platforms and wood shavings were less fearful of a human hand inserted into their cage compared to those living in a small cage with a single suspended platform, with or without wood shavings.

Based on their own experience caring for colonies of **naked mole rats** for many decades, Smith and Buffenstein (2021) advocated enrichment that promotes these animals' natural behaviors of building and extending burrow systems, foraging, and engaging in housekeeping activities. To simulate the natural experience of encountering a variety of stimuli when excavating a burrow in the wild, naked mole rats should be given various types of fruits, vegetables, and substrates (e.g., sandpaper, tissue paper, pumice stones, corn husks). The authors also recommended blocking their tunnels with items such as large yams or paper towels. However, they noted that each colony responds differently to various forms of enrichment, so it is important to monitor the animals closely after a new item is introduced to ensure that it isn't causing them stress. Signs of stress include production of softened fecal pellets and an increase in aggression or audible antagonistic communication.

Rabbits

STRUCTURAL ITEMS

Rabbits in social pairs typically spend most of their time together (Huls et al., 1991; Whary et al., 1993), but sometimes prefer to be alone (Held et al., 1995). Thus, it is important to give them adequate space and structural enrichment to provide opportunities for separation from conspecifics. Cardboard boxes or huts, for example, allow them to withdraw from conspecifics, perceived threats, or exposure to light. Rabbits will also commonly sit on top of them as a lookout (Hansen & Berthelsen, 2000). Overturned rat cages can also serve as lookout perches ("Rabbit Enrichment Items," 2019).

A descriptive study observed group-housed female New Zealand white rabbits in a large floor pen, where they were provided with 14 different enrichment items, one at a time. Rabbits engaged the most with a bed of straw or paper wool, a repurposed wooden cable drum that served as a table, and cabbage hung from a washing line above the pen (Figure 16; Pearson & Gant, 2018). Focusing on hanging enrichment, Bozicovich et al. (2016) demonstrated that group-housed male and female rabbits

Figure 16: In Pearson and Gant (2018), female rabbits group-housed in a large floor pen engaged the most with a bed of straw or paper wool, a repurposed wooden cable drum that served as a table, and cabbage hung from a washing line above the pen.

Figure 17: In Coda et al. (2020), a hanging wire ball containing a bell and timothy hay promoted exploratory behavior and reduced self-grooming in rabbits.

from the Botocatu genetic group interacted more with wooden sticks (pine, eucalyptus, or bamboo) compared to PVC pipes when these items were suspended with wire from the cage ceiling. In a follow-up experiment, rabbits were provided with two hanging sticks of eucalyptus; this resulted in a decrease in self-grooming in both sexes and an increase in aggressive behavior in males (although the number of individuals with skin wounds was actually lower compared to the unenriched group). Males also had larger visual and sensorial areas in their brains and increased brain weight. The incidence of stereotypies was not changed.

Coda et al. (2020) assessed the behavior of male and female New Zealand white rabbits when they were briefly exposed to one of three novel objects: a hanging wire ball containing a bell and timothy hay, a plastic digging bin containing corncob bedding and hay, or a handmade destructible paper box filled with hay (Figure 17). All three objects were engaging for the rabbits and promoted exploratory behavior, but the hanging ball and destructible box also reduced self-grooming and were reported to be less labor intensive for staff compared to the digging bin.

Poggiagliolmi et al. (2011) compared chewing behavior in single-housed male New Zealand white rabbits provided or not provided with one type of chew toy at a time (cardboard rings, a cardboard roll, or a rubber ball with bell inside). Rabbits with or without the chewing items spent an equal amount of time chewing on the bars of the cage, but those with the chewing items also chewed on those items (and therefore, spent more total time chewing), which may help prevent malocclusion. The three types of chewing items were used equally by the rabbits. In rabbits raised for meat production, chicory pulp gnawing blocks hung from the cage lid were found to be suitable cage enrichment for female rabbits in situations where caretakers are concerned about disease transmission from items on the cage floor (Maertens et al., 2013).

A few studies investigated the potential for various enrichment items to mitigate aggression among group-housed rabbits; these studies were conducted with rabbits raised for meat production in commercial settings, but their findings could be useful for rabbits housed in laboratories. Rommers et al. (2014) assessed newly formed groups of four rabbit does and their kits (hybrid strain derived from New Zealand white) in combinations of different treatments: familiarity with the cage before grouping; straw provided on the cage floor and from a hanging feeder; and hiding places provided in the form of a hanging plastic tunnel and an elevated platform. Aggression was seen in all treatments, but rabbits housed with hiding places had the lowest prevalence of severe injuries. There were no differences in aggression-related injuries between rabbits grouped in cages already scent marked by one rabbit versus those grouped in clean, unfamiliar cages, suggesting that hierarchy-related fighting may occur after regrouping regardless of established territories. Van Damme et al. (2024) also assessed newly formed groups of four rabbit does and their kits (Hyla strain) in different treatments: All cages were furnished with elevated platforms, wood gnawing blocks, and nest boxes with nesting material (flax and wood shavings). Control-group cages contained no additional enrichment, while in other groups, the cages contained either pressed alfalfa blocks as "distraction enrichment," vertical panels that separated the cage into four areas, or both alfalfa blocks and vertical panels. Similarly to Rommers et al. (2014), Van Damme et al. found high rates of aggression among all groups, but groups with access to the alfalfa blocks—either with or without the vertical panels—had fewer does with skin injuries. In another study, male and female group-housed Hyplus rabbits used a long, elevated platform as a hiding place and spent more time rearing and resting in a stretched (relaxed) position compared to those without the platform. Large plastic tunnels were not used very much and did not confer measurable welfare benefits. Rabbits provided with the elevated platform had higher rates of aggression-related injuries, but this result was

confounded by group sizes, with rabbits in the control group housed in groups of 27 and rabbits with platform access housed in groups of 36, increasing the probability of aggressive interactions (Trocino et al., 2019). Finally, mixed-sex groups of cross-bred rabbits (50% New Zealand white, 25% Californian, 25% large butterfly) were housed in groups of eight in various cage sizes (to achieve different stocking densities) with or without a wooden U-shaped structure (used for sheltering, gnawing, and resting off the wire floor of the cage) and subjected to an acute stressor (transport for 30 minutes). Stocking density had no effect on any measures, except increased lying and decreased sitting or standing in the smallest cages. Fecal glucocorticoids (a marker of stress) were lower in cages with the U-shaped structure, both before and after transport. The structure also decreased cage manipulation and increased social behaviors (e.g., sniffing or grooming another rabbit). Aggression was seldom seen and was unaffected by stocking density or the presence of structural enrichment (Buijs et al., 2011). In summary, it appears that structural items that allow group-housed rabbits to gnaw, shelter, and rest above the floor offer some benefits, though their role in mitigating aggression is inconclusive.

FEEDING AND FORAGING ENRICHMENT

Several creative foraging enrichment ideas for rabbits have been proposed by participants in AWI's online discussion forum (LAREF). Participants touted the benefits of timothy hay cubes, dried fruit/veggie mix, wooden cubes soaked in pineapple juice, and papaya tablets. They also highly recommended hiding treats inside destructible/shreddable items such as paper bags or cardboard tubes to promote foraging, or providing recycled cardboard boxes (such as empty glove boxes) stuffed with shredded paper or hay to create a dig box ("Rabbit Enrichment Items," 2019). These types of destructible foraging resources may encourage more active engagement with enrichment; they may also be particularly useful at distracting post-operative animals who need to focus on something other than their incisions. As another creative example of inexpensive foraging enrichment, autoclaved recycled cardboard can be cut into six interlocking circles and stuffed with hay or other treats (Figure 13; Buchanan, 2020).

ENVIRONMENTAL COMPLEXITY

Worlds et al. (2020) described an enrichment program for rabbits who were used in safety testing or preclinical trials in which the animals received a hut, hanging toy, and toy on the cage floor and were routinely provided timothy hay, apple slices, or leafy greens. Once the rabbits were acclimated to the facility, they were provided access to a sanitized play area (dog-pen caging or dedicated runs) once a week for

1–2 hours. Staff and researchers reported that the animals seemed calmer, easier to handle, and had fewer veterinary health concerns. In another enrichment program within an antibody production facility, female New Zealand white rabbits were housed in groups of 20 within large pens and provided large shelters in addition to wooden blocks, various cardboard structures, tray liners, and dumbbells (Figure 18).

All the resources were used by the rabbits, with destructible objects facilitating more social interactions between rabbits, and other items such as dumbbells being used for scent marking (Davenport et al., 2017). At another facility, rabbits who are single housed (and are therefore required to receive extra environmental enrichment) are provided with foraging boxes, digging boxes, huts, lofts, gnawing devices, floor exercise, and positive human interaction (Mantz & Pugerud, 2024).

Figure 18: In Davenport et al. (2017), destructible objects facilitated social interactions between pen-housed female rabbits, while other items such as dumbbells were used for scent marking.

AUDITORY ENRICHMENT

Peveler and Hickman (2018) found that single-housed male New Zealand white rabbits had decreased cortisol levels when played daily soothing music from a commercially available CD of music for rabbits. When the music was removed, cortisol levels returned to baseline levels, indicating that soothing music may help reduce stress. LAREF participants have indicated that rabbits really seem to enjoy throwing around items that make noise, such as canning jar lids (with the rubber removed) or stainless steel bowls ("Rabbit Enrichment Items," 2019).

KEY TAKEAWAYS: ENVIRONMENTAL ENRICHMENT

- "Environmental enrichment" can be a misnomer; the term is often used to refer to *any* resources that are provided to animals beyond bare cages, even when those resources are merely serving to meet animals' most basic needs (e.g., nesting material for mice). The typical laboratory cage is poorly provisioned and does not adequately address animal needs. We therefore encourage readers to avoid thinking of cages containing any one additional component as "enriched."

- Increasing environmental complexity by providing multiple types of enrichment and additional space markedly improves welfare for **rodents** and **rabbits** alike. It is important to note, however, that providing additional resources without additional space may overcrowd the cage, potentially negating the benefits of additional resources if the animals' ability to move about is restricted. Compared with barren or poorly resourced cages, rodents housed in environments with more space and a variety of resources tend to have increased sociability, learning, memory, and exploratory behaviors, as well as reduced stereotypies, barbering, anxiety, and depression.

- Improving the home environment through the provision of additional space and resources does not adversely impact experimental outcomes. A growing body of evidence indicates that increased environmental variability does not alter the variability of experimental results. Providing environmental enrichment also has the added benefit of reducing compassion fatigue for laboratory personnel.

- Although improving animals' permanent housing is preferred, in situations where that is not possible, **rats** and **mice** can be temporarily placed in playpens to increase opportunities for natural behavior. This can often be accomplished using resources already available within a facility, such as repurposed cages for larger species, bedding, cardboard boxes, tunnels, and nesting material or paper towels. However, permanent discontinuation of access to playpens may be perceived as a significant loss by the animals, and this can induce negative emotional states even more pronounced than if animals had never received this type of enrichment. Therefore, any provision of enrichment that subsequently will be permanently removed should be considered with caution.

- Adding enrichment is sometimes avoided due to fears of increased aggression in the animals. However, in many cases, aggression stems from the stress of husbandry practices or experimental manipulations rather than the presence or absence of structural objects within the cage. To reduce aggression among

mice—particularly males—it is important to minimize animal stress (e.g., by avoiding tail handling or social regrouping).

- → It is especially important to provide **mice** with appropriate nesting material, as mice are ubiquitously cold stressed in laboratories set to temperatures that are comfortable for humans. To avoid cold stress and its many physiological and emotional consequences, at least 6 grams of nesting material should be provided to allow mice to keep warm inside a nest. Larger quantities of nesting material (8–12 g) are recommended, especially for females or mice in IVCs. Mice also benefit from running wheels, foraging puzzles, shelters, and chew sticks.

- → **Rats** should be socially housed where possible; rats also benefit from nesting material, shelters with a single opening, running wheels, gnawing items, foraging puzzles, elevated platforms or hammocks, and larger cages.

- → **Other rodents** can also benefit from social companionship, nesting material, shelters, running wheels, foraging puzzles, and gnawing items.

- → Providing **rabbits** with more space in larger runs (e.g., dog pens) can promote activity and exploratory behavior. Female rabbits can be housed in large social groups in this environment. Aggression may be reduced in socially housed rabbits by providing structural enrichment that allows for voluntary social separation. Rabbits also benefit from gnawing opportunities and foraging puzzles.

- → There are many options for cost-effective, easy-to-implement DIY enrichment items, such as converting glass pasta sauce or jelly jars into shelters for **mice**; modifying PVC pipes for use as tunnels, shelters, or hammocks for **rodents**; and creating foraging puzzles out of toilet paper rolls or paper bags stuffed with hay and treats for **rodents** and **rabbits**.

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Abnormal Behavior

What Is It, And Why Does It Matter?

Abnormal behaviors are behaviors that are unusual in quality (e.g., only seen in captivity) or quantity (e.g., performed excessively). These behaviors tend to develop as animals attempt to achieve a goal that cannot be met in an environment that is species inappropriate (Winnicker et al., 2016). As such, abnormal *behavior* is often a misnomer, because it is the *environment* that is abnormal, and the behavior is simply a normal animal's attempt to cope with that environment. Better terms would be "maladaptive" in the case of natural but exaggerated behaviors, such as excessive aggression, infanticide, and food grinding; or "malfunctional" in the case of behaviors that have become pathological, such as stereotypies and barbering or fur chewing. Some behaviors can truly be abnormal if they stem from an underlying pathology; for example, as a result of a knockout gene (Winnicker et al., 2016). In this book, we refer to all these behaviors as "abnormal" (rather than maladaptive or malfunctional) to be consistent with how they are commonly referred to in the literature.

The prevalence of abnormal behaviors in laboratory rodents is alarming. In mice, one study documented that 75% of pairs of female CD1 and 100% of pairs of female C57BL/6/JRcc mice housed with nesting material performed stereotypic bar mouthing, with this and other stereotypies occupying, on average, 19% and 15% of active time in these two strains, respectively (Novak, Stojanovski, et al., 2016). In barren cages, stereotypies can occupy as much as 50% of the animals' active time (Gross, Engel, Richter, et al., 2011). In gerbils, 100% of pairs housed with a dark nest box were observed performing stereotypic corner digging, and 94% were observed performing stereotypic corner jumping (Habenicht et al., 2022).

Abnormal behaviors can represent a serious welfare concern because they likely arise out of frustration or distress, are often linked to other indicators of poor welfare, and can lead to pathology, illness, or injury (Mason & Latham, 2004; Winnicker et al., 2016). Moreover, by definition, animals who perform maladaptive or malfunctional behaviors are not representative of the "normal" population of their species or strain. Indeed, stereotypies in mice closely match biomarkers of stereotypies in multiple human disorders, and barbering was shown to be a highly specific model of trichotillomania (a human mental disorder characterized by pulling out one's own hair; Garner et al., 2011). This means that mice performing stereotypies or barbering may be better suited as models of specific disorders, not as "normal" animals.

The present chapter synthesizes publications that are explicitly focused on causes and prevention of abnormal behaviors. Abnormal behaviors are also discussed in other chapters (e.g., **Environmental Enrichment**) if their prevalence was recorded as a study outcome.

Summaries of Current Refinement Research

Mice

POSSIBLE CAUSES

In one study, the role of frustration was investigated as a potential cause of stereotypic behavior. Latham and Mason (2010) single housed newly weaned male and female ICR CD1 mice in shoebox cages that either contained bedding and nesting material (standard condition) or bedding, nesting material, nest box, plastic tunnels, and a weekly rotating novel object (enriched condition). Mice in both conditions developed stereotypies, but those in the standard condition developed significantly more. When the mice were 3 months old, all were rehoused into the standard condition. Mice who had been downgraded from the enriched condition became substantially more stereotypic than those who had always lived in the standard condition. They also worked harder to gain access to enrichment or an empty cage that contained food. Moreover, within the downgraded treatment, individuals who were most motivated to access enrichments had higher levels of the stress hormone corticosterone, and males (but not females) who had higher levels of corticosterone performed more stereotypies. The authors concluded that frustration seemed to play a causal role in the exacerbation of stereotypies after loss of enrichment. This study provides evidence that (1) an enriched shoebox cage is not enough to prevent the development of abnormal behavior, and (2) the removal of enrichment leads to more abnormal behavior than never providing it—a caution against providing enrichment early in life if it cannot be provided for the duration of the animal's life.

Cage-induced stereotypic behavior has been linked with altered affective states in some strains of mice, but these effects may vary according to the type of stereotypy performed. Novak, Bailoo, et al. (2016) trained female CD1 and C57BL/6 mice in

a judgment bias test (a method to determine if animals behave optimistically or pessimistically, as an indicator of mood) and found that CD1 mice with higher stereotypy levels displayed more pessimistic choices; specifically, backflipping was associated with negative judgment bias, but bar mouthing and twirling were not. There was no relationship between stereotypies and mood in C57BL/6 mice. Another study using the same strains but a different type of judgment bias test found different results: In this study, mice of both strains who performed more stereotypies were more optimistic; however, there was no effect when looking only at bar mouthing, which was the most prevalent type of stereotypy (Novak, Stojanovski, et al., 2016). After mice were subjected to an unpredictable chronic mild stress protocol that induced more pessimistic responses in the judgment bias test, bar mouthing in CD1 mice increased. No change was seen in other types of stereotypies, nor for any of the stereotypies in C57BL/6 mice. These results reveal that each type of stereotypy may have a different cause and a different welfare implication, and that these effects may vary between strains.

There is some evidence that litter size predicts the development of stereotypic behavior in adult female mice. Bechard et al. (2012) found that females (but not males) raised in larger litters were significantly more likely to develop stereotypic behavior as adults. This finding was true in the three strains tested (C57BL/6N, C57BL/6J, and CD1). Stereotypic behavior was unrelated to pup weights at weaning, indicating that the increased likelihood of stereotypies found in larger litters may have been related to the social environment during development rather than differences in maternal care or milk transfer. A different study examined the effects of weaning age (ranging from 14 to 30 days of age) and social housing conditions (single vs. groups of three) on the development of stereotypic behavior in adult male and female RjORL:SWISS mice (Bailoo et al., 2020). Regardless of weaning age, single-housed mice exhibited more stereotypic behavior than group-housed mice. Weaning age, however, had little effect on stereotypic behavior and other measures.

One study attempted to disentangle the effects of environmental complexity, environmental novelty, or access to specific resources on the development of stereotypic behavior. Three groups of pair-housed male ICR CD1 mice were housed with 2 grams of cotton wool nesting material. Two of the three groups also had access to one of three possible shelters and one of three possible climbing structures (resulting in nine possible combinations). Some pairs had access to only one of these possible shelter/climbing structure combinations throughout the 9-week study, while others received a different combination every week (Gross, Engel, & Würbel, 2011). Housing condition had no effect on the rate of stereotypic behavior performed

at 12 weeks of age, with mice in all treatments spending an average of 19% of their active time performing stereotypies. Using female pair-housed mice of the same strain in a different study, a subset of these same authors found comparable levels of stereotypies (16% active time) in enriched-housed mice (nesting material, shelter, climbing structure, and access to another highly enriched cage three times per week for 10 hours), but much lower than the levels of stereotypies (50%) in mice housed in barren cages (Gross, Engel, Richter, et al., 2011). The authors suggested that nesting material may be the most effective means to mitigate the development of stereotypies in mice, and that additional structural complexity or novelty in shoebox cages were not effective at preventing their development.

Food grinding is another behavior considered to be abnormal in mice, although its specific cause is not well understood and may simply reflect a normal part of optimal food intake strategy (Cameron & Speakman, 2010). To assess what might motivate mice to engage in food-grinding behavior, Pritchett-Corning et al. (2013) provided group-housed female CD1 mice with a nylon chew object (I Chew) or sunflower seeds for 4 weeks. Both treatments reduced food grinding while they were provided. When the nylon chew was removed, food grinding returned to baseline levels; when sunflower seeds were removed, mice maintained decreased food grinding for 1 week (food grinding was not assessed beyond 1 week after treatment removal). Additionally, regardless of treatment, mice who weighed more engaged in less food grinding, and the grinding consisted of discarding fiber and carbohydrates and ingesting only the fat. These results suggest that food grinding may be motivated by nutritional and energy requirements rather than a need to gnaw. This suggestion is supported by MacDuff et al. (2019), who found that mice provided with the type and amount of nesting material that produced the best quality nests (i.e., those that offered best heat insulation) also engaged in the least food grinding. Furthermore, Gaskill et al. (2025) replicated findings of Pritchett-Corning et al. (2013) that mice provided with higher-fat diets engage in less food grinding; in the new study, mice reduced grinding when provided with either sunflower seeds or a pelleted diet supplement formulated to approximate the macronutrients of sunflower seeds. Thus, scientific evidence suggests that food grinding is motivated by nutritional rather than behavioral needs.

PREVENTION/REDUCTION STRATEGIES

Various types of environmental enrichment can help prevent or reduce the incidence of abnormal behaviors in mice. Moody et al. (2021) found that young group-housed female C57BL/6NCrl mice provided with a facial tissue and a puck of compressed

nesting material (8 grams of crinkle paper) showed no evidence of barbering within 15 days, whereas mice provided with only a facial tissue, or a facial tissue with 8 grams of crinkle paper scattered in the cage, did (Figure 1). Mice spent more time manipulating the nesting material presented in compressed format, which may have helped reduce the time spent performing abnormal behaviors.

Complex housing may also help decrease barbering in mice. Newly weaned male and female C57BL/6J mice were socially housed in either standard shoebox cages with a cotton Nestlet and a shelter, or in larger cages with a shelter and one other item rotated bi-weekly (tunnel, plastic container, or nylon chew). At 4 months of age, only mice in the standard housing had developed barbering (visible in one-third of cages). At 6 months of age, almost two-thirds of the mice in the standard housing showed evidence of barbering, compared to about one-third of those in enriched housing. Therefore, while more complex housing did not prevent barbering altogether, it delayed its onset and decreased its prevalence and overall severity (Bechard et al., 2011).

Figure 1: In Moody et al. (2021), a puck of compressed nesting material was more effective at preventing barbering in female C57BL/6 mice than the same amount of crinkle paper scattered in the cage.

Once established, stereotypies can be challenging or impossible to abolish, but they can be reduced. Tilly et al. (2010) raised pair-housed female C57BL/6 mice in shoebox cages furnished with nesting material and a shelter. When, later in life, these mice were moved to larger cages that contained cardboard tubes, shelters, nesting material, gnawing sticks, a hammock, and their original shoebox cage, stereotypic behavior was reduced in every mouse. However, the new environment was less effective at reducing stereotypic behavior in middle-aged mice (10–11 months old) with more established stereotypies, compared to younger mice (6–7 months old). These older mice were also less motivated to access enrichment, suggesting that the rewarding aspects of environmental enrichment were diminished for these mice. Therefore, to mitigate the negative effects of substandard environments, better-resourced environments should be provided as early as possible.

The use of enrichment in the form of olfactory cues from other mice may also help decrease the prevalence of abnormal behaviors. In one investigation, 8-week-old C57BL/6J female mice who performed stereotypies but showed no evidence of barbering were group housed under one of two conditions: At weekly cage changing, "sentinel" mice received a 50:50 blend of clean and dirty bedding (the latter mixed from other mice in the colony), while control mice received only clean bedding (Müller et al., 2022). After 12 weeks, sentinel mice demonstrated less barbering and bar mouthing and more social grooming. Weight gain was the same in both groups. The authors suggest that the soiled bedding may serve as a form of olfactory enrichment. A different study found that female ICR sentinel mice who received only dirty bedding at weekly cage changing gained weight more slowly than mice provided with clean bedding (Merley et al., 2022). The differences between these studies could be due to differences in the degree to which the sentinel mice bedding was soiled (50% versus 100%) or to the different strains.

Regarding food grinding, one descriptive article (Oralman, 2020) suggested that placing a "cruncher barrier"—a piece of cardboard or pulp paper (e.g., Bio-Huts for Rats)—over the nest discourages mice from food grinding (Figure 2). The cruncher barrier serves multiple purposes: It protects the nest from becoming submerged in crumbs, provides an alternative substrate to destroy, and increases access to nesting material (once the hut is shredded). Technicians viewed this intervention as beneficial for reducing food grinding behavior and the need for food top-ups. Another study found that group-housed female CD1 mice engaged in significantly less food grinding after a running wheel was added to their cage (Skurnack et al., 2024). When the wheel was removed, grinding dramatically increased for the first 4 days before returning to the same levels observed during wheel access. However, the authors

Figure 2: Placing a "cruncher barrier" over a mouse nest can discourage mice from food grinding (Oralman, 2020).

also documented that daily food usage increased by about 5 grams with every 1% increase in relative humidity, and relative humidity happened to be highest in the days after wheel removal (see also Cordeira (2023) for more evidence that mice find food more palatable at higher humidity). Therefore, wheel access does seem to reduce food-grinding behavior, but the effect of wheel removal on food grinding is unclear. Other forms of environmental enrichment may be less effective at mitigating food grinding. Using a small sample size of two cages per treatment, Garcia et al. (2021) provided a gnawing block or sunflower seeds to group-housed CD1 mice, some of whom engaged in food grinding and some of whom did not. Mice who engaged in food grinding trended slightly toward less food grinding for the first 4 weeks after the addition of either a gnawing block or sunflower seeds. Of the mice who were not previously grinding, only those who received sunflower seeds did not develop food grinding by the end of the 8-week observation period. Cameron and Speakman (2010) found that adding shelters (hut, igloo, tunnel), a gnawing block, or a crawl ball for a single day did not reduce food grinding in female MF1 and C57BL/6 mice, even though these items did increase mouse activity. It is worth noting that the mice were single housed at relatively low temperatures (68 °F (20 °C); see chapter on **Environmental Enrichment**), suggesting that these mice may have been highly motivated to select for higher caloric intake to meet energy requirements. Finally, one study found that adding an unraveled Diamond Twist (paper nesting material) increased food grinding in single- but not pair-housed female Swiss Webster mice (Zawacki et al., 2024).

Other Rodents

Fur chewing is an abnormal behavior commonly occurring in captive **chinchillas**. Łapiński et al. (2020) compared single-housed male chinchillas who exhibited fur chewing to those who did not fur chew. Using an infrared thermography camera to measure body temperature, they found that fur-chewing chinchillas had 21% higher heat loss than non-fur-chewing animals. Fur-chewing animals also had increased feed intake, likely due to their decreased fur insulation. This is a welfare concern if these animals are experiencing heightened thermal stress and a research concern if they are metabolically different from normally behaving animals.

Fur chewing is the most commonly reported abnormal behavior in chinchillas due to its impact on the fur-farming industry, but other abnormal behaviors have also been documented. In a commercial farm setting, 10 chinchillas housed individually in barren conditions were filmed for 24 hours, and all were observed performing at least one abnormal repetitive behavior, including fur chewing, bar mouthing, repetitive scratching at the cage, and backflipping (Franchi et al., 2016). These behaviors were more common at night than during the daytime. Chinchillas who did not have any signs of fur chewing were all observed performing other abnormal behaviors.

KEY TAKEAWAYS: ABNORMAL BEHAVIORS

- Abnormal behaviors may occur for a variety of reasons. However, in most cases, abnormal behaviors are indicative of inadequate living conditions and represent a welfare concern. Abnormal behaviors may increase due to thwarted attempts to perform motivated behaviors.
- Stereotypies can be challenging or impossible to eradicate once they are established, at which point they may become habitual rather than reflective of current negative affective states.
- Downgrading animals from well-resourced to poorly resourced conditions should be avoided, as this can exacerbate abnormal behavior.
- In **mice**, each form of stereotypy (e.g., bar mouthing, twirling, backflipping) may have a different cause and a different welfare implication, and these effects may vary between strains.
- Single-housed **mice** tend to perform more stereotypies than group-housed mice. Mice exposed to poorly resourced environments tend to exhibit higher rates of stereotypic behavior than mice who are exposed to larger, more complex environments.
- Providing appropriate nesting material appears to be the most effective way to reduce stereotypic behaviors in **mice**. Providing olfactory cues from other mice via bedding has also been suggested as an effective strategy. Food grinding in mice may be indicative of nutritional deficiencies rather than abnormal behavior. Providing a gnawing device and/or sunflower seeds can reduce food grinding.
- Abnormal behaviors for **chinchillas** include fur chewing, bar mouthing, repetitive scratching at the cage, and backflipping. These are commonly observed when chinchillas are housed in barren conditions. Chinchillas who chew their fur have lower body temperatures and consume more food due to heat loss, which raises concerns both for the welfare of the animals and the validity of the data obtained from them.

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Human-Animal Interaction

What Is It, And Why Does It Matter?

Most rodents and rabbits are naturally fearful of humans, whom they perceive as large predators. Wild rats, for example, adjust the times of day they spend outside their burrows to avoid peaks in human activity: Around a busy market in Malaysia, rats were recorded to retreat back into their burrows just as the market set to open (Oyedele et al., 2015), and on a university campus, rats exhibited a gradual shift toward diurnality in the weeks when fewer students were around and abruptly reverted back to nocturnality when the students returned (Recht, 1982).

In laboratories, regular contact with humans is unavoidable. It is therefore crucial to establish a positive relationship with rodents and rabbits early on, so that we can become a source of enrichment rather than anxiety. This will not only contribute to good welfare but also ensure that our "control" animals aren't inadvertently models of chronic anxiety.

Simply habituating the animals to our presence is not enough. A few studies have shown that laboratory rats whose interactions with humans were limited to routine husbandry procedures produced 22-kHz vocalizations indicative of anxiety after being touched (Brudzynski & Ociepa, 1992) and showed increases in corticosterone (Armario et al., 1986; De Boer et al., 1990), noradrenaline (De Boer et al., 1990), heart rate, and blood pressure (Baturaite et al., 2005; Sharp et al., 2002, 2003) when gently lifted from their cages. Habituation and desensitization are more effective when paired with a positive reinforcer, such as a food reward or other positive activity, such as play. Habituation and training require some time investment at the front end of a study, but the consensus is that there is a "return on investment" as the study progresses, because working with animals who are calm and cooperative takes less time. Importantly, working with calm and cooperative animals is less stressful for the animals and the experimenters, leading to fewer stress-related mistakes and ensuring that the animals are more demonstrative of a "normal" population they are intended to represent.

Routine husbandry procedures occurring outside of experiments can have profound impacts on animal welfare, whether they are acutely stressful one-time procedures (e.g., ear notching) or regularly occurring events (e.g., cage changing). Handling and restraint performed during a study also have the potential to cause incredible welfare harm if performed without care. In the context of research, such harm has the added consequence of altering physiological and behavioral parameters that confound—

or even invalidate—experimental outcomes. Assenmacher et al. (2022) performed postmortem examinations on 864 mice of various strains who had undergone physical restraint, blood sampling, and injections during prior studies. A comparison group of 136 mice were only handled during cage changing. The findings are extremely troubling: Of the mice subjected to manual handling and restraint, 61 had at least one form of traumatic bone or articular damage such as fractured ribs, fractured cervical vertebrae, or dislocated joints, compared to one mouse from the comparison group. In addition, 133 had signs of trauma in or around the eye related to retro-orbital blood collection, 39 had trauma related to maxillary or facial vein blood collection, and 92 had penetrating abdominal trauma and pathological changes related to receiving intraperitoneal (IP) injections. It is not acceptable for mice to suffer from undetected, accidental fractures, dislocations, and other trauma occurring during routine experimental procedures. Structured hands-on training of researchers and recurring assessments of technique by veterinarians are crucial for reducing adverse events related to restraint and basic experimental procedures. In many cases, having procedures carried out by experienced veterinary staff is a refinement over having them performed by a student or researcher with little technical expertise in animal procedures.

Even when handling and restraint do not result in physical trauma, each such interaction can shape how the animal will perceive future interactions with humans, and thus it is important to be mindful of the method and approach used during *each interaction*. At minimum, the goal should be to minimize the negative impact of any procedures known to be stressful; ideally, each interaction should be seen as an opportunity to promote positive welfare and strengthen the human-animal relationship.

For example, proper hydration is essential for overall health and to reduce risks associated with medical interventions. Hydration methods can vary in their invasiveness, and the method used can dramatically change the animal's experience and relationship with the experimenter. A common hydration method is to administer an intravenous or subcutaneous injection that often necessitates physical restraint and needlestick that can cause discomfort. A refined approach is to train the animal to voluntarily drink from a blunt-tip syringe. Such "cooperative handling" gives animals a sense of control, which not only results in lower stress but also eliminates the need for physical or chemical restraint; both of these factors contribute to greater validity of scientific results by eliminating unintended study confounds (Graham et al., 2012).

An ideal approach, however, would be to use this opportunity to engage the animals in a game. An example of this with rats is pea bobbing (or "pea fishing")—an activity promoted by Dr. Melanie Graham of the University of Minnesota and designed to

Figure 1: "Bobbing for peas," a fun alternative to subcutaneous fluid administration, effectively eliminated porphyrin staining around the nose, decreased avoidance behaviors, and led to a shorter latency for rats to approach caretakers.

improve hydration (Figure 1). Peas are a rat-safe vegetable with a high water content of about 80%. Carrots—which have a water content of about 90%—can also be used in place of, or along with, frozen peas. (In moderation, peas and carrots have not been found to cause diarrhea, unlike other high-water-content vegetables such as cucumbers or celery.) In this activity, a shallow bowl is filled with room temperature water and frozen peas. This encourages rats to engage in natural foraging behavior by reaching for, dipping into, and grasping the floating and sinking peas. This setup offers sensory enrichment and presents a food-based puzzle, effectively combining hydration with cognitive stimulation. Dr. Graham's group observed that implementing this approach to enhance hydration after certain procedures—as an alternative to subcutaneous fluid administration—effectively eliminated porphyrin staining (red substance indicative of stress or sickness) around the nose, decreased avoidance behaviors, and led to a shorter latency for rats to approach caretakers. Hydration strategies that align with species-specific behaviors not only achieve effective fluid replacement but also minimize disruptions to the animals, provide enrichment, and strengthen the human-animal bond, thereby reducing stress and improving overall welfare.

A positive relationship with animals can be inferred when animals voluntarily approach or seek contact with the human, show behavioral signs of positive anticipation before contact with the human, and exhibit a relaxed posture or other indicators of pleasure during or after interaction with the human (Rault et al., 2020). A positive human-animal relationship in a laboratory context benefits animal welfare by buffering the stress of aversive procedures; it also leads to easier handling and increased satisfaction for the human caregiver (Rault et al., 2020).

Summaries of Current Refinement Research

Mice and Rats

Since 2015, the RISE Research Institutes of Sweden have actively worked to improve the welfare of mice and rats in toxicology studies. The cornerstone of their efforts is to ensure that before the study begins, every rodent arriving at the facility—regardless of how long they are expected to stay—is gently handled and habituated to the procedures they will be exposed to during the study (Bengtsson & Eriksson, 2020). All animals are handled and socialized beginning on the first day they arrive at the facility. The time investment is minimal: Only 1–2 minutes repeated five times is usually sufficient. Habituation is focused on familiarization with a Vetbed (a plush surface used for handling) and the specific procedures each animal will be subjected to. For example, animals who will have blood sampled from the saphenous vein are habituated to restraint grip and the noise of hair clippers; animals who will have blood sampled from the tail are habituated to their tail being touched and gently picked at; and animals who will be restrained for prolonged periods in the course of inhalation studies are trained to freely enter the restraint tube, where they will find a treat (Figure 2). The goal is to increase trust between the animals and handlers, and to reduce stress-related mistakes that may arise from working with an uncooperative animal. Additionally, mice and rats are given positive reinforcement at the end of training and experimental procedures—for example, time to explore a larger "observation box" with conspecifics. Bengtsson and Eriksson reported that working with calm and cooperative animals saves time (animals do not struggle, and staff make fewer stress-related mistakes), is better for the animals and personnel, and is better for scientific outcomes, since calmer animals are more "normal" physiologically and behaviorally, and therefore more representative of the humans for whom they serve as models. An overview of these methods is available on the RISE Institutes of Sweden YouTube channel (https://perma.cc/5A6S-NFSV).

Professor Emma Robinson of Bristol University launched the 3Hs Initiative: Housing, Handling, and Habituation (Bartlett et al., 2024). In a webinar, she explained that if an animal doesn't like interacting with a human, then every interaction is a negative experience, and those negative experiences accumulate. Conversely, if an animal enjoys interacting with a human, then every interaction contributes to the accumulation of positive experiences for that animal. These accumulated experiences

have important consequences for the animal's welfare. To address this, one of the core aims of the 3Hs Initiative is to reduce the use of stressful physical restraint in rats and mice undergoing oral dosing and IP and subcutaneous injections. Several high-quality videos demonstrating the steps involved in habituation to handling and non-aversive restraint are available on the 3Hs Initiative website, along with supporting scientific evidence (https://perma.cc/JH6Q-8QUS).

Figure 2: Before a study begins, animals should be habituated to the procedures they will be exposed to during the study. For example, animals who experience tail vein sampling should be habituated to their tails being handled and gently picked at, and animals who will be restrained for prolonged periods in the course of inhalational studies should be trained to freely enter the restraint tube, where they will find a treat.

Mice

HANDLING AND RESTRAINT

Hurst and West (2010) conducted a seminal study demonstrating that the standard method of picking up mice by the tail induces anxiety and avoidance of the handler's hand, whereas picking up mice with their home-cage tunnel (i.e., tube) or an open hand (referred to as "cupping") induces lower anxiety and increases voluntary approach to the handler. Specifically, compared to mice who were tunnel handled or cupped, tail-handled male and female C57BL/6, BALB/c and ICR(CD1) mice showed greater urination and defecation during handling, fewer open arm entries, and more

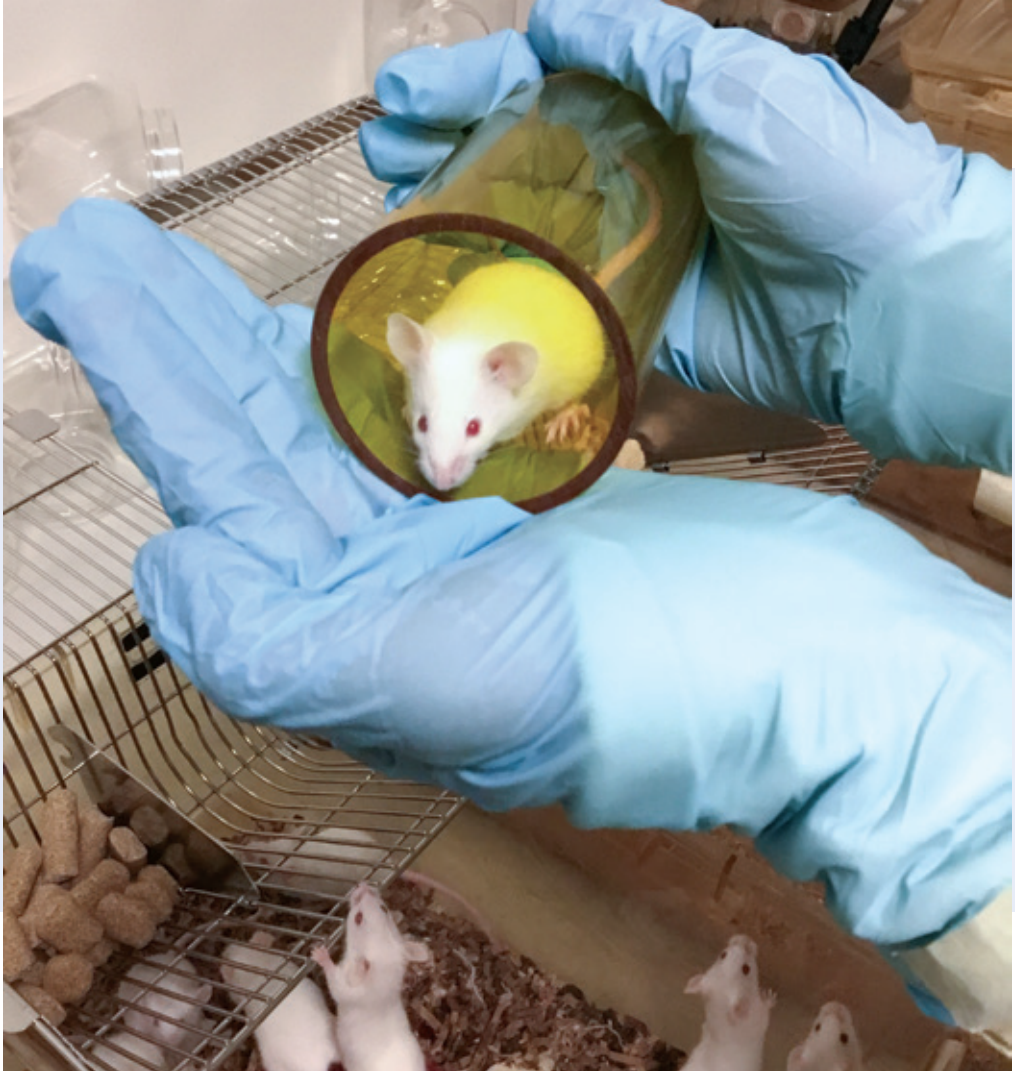

Figure 3: Tunnel handling is a humane alternative to aversive tail handling in mice.

"protected stretched attend postures" (stretching the head forward from a protected area—considered a sign of anxiety) in the elevated plus maze test, and reduced voluntary interaction with the handling device both before and after handling. The authors noted that aversion to tail handling is likely a naturally selected response, since capture and lifting by the tail is one typical way predators catch mice. A subsequent study with male and female C57BL/6JOlaHsd and ICR(CD1) mice demonstrated that the positive effects of tunnel versus tail handling are similar whether one uses the animals' home-cage tunnel or a separate tunnel shared by all cages (Gouveia & Hurst, 2013). Nevertheless, after several handling sessions, C57BL/6JOlaHsd mice interacted more with the handling device when it was the home versus a shared tunnel; ICR(CD1) mice showed no preference. This suggests that some strains may prefer handling with their home tunnel rather than a shared tunnel (Figure 3).

Many more experiments have since demonstrated the negative effects of tail handling on mouse welfare and behavior. For example, Clarkson et al. (2018) demonstrated that, in addition to showing lower voluntary interaction with the handler and higher anxiety in the elevated plus maze test, tail-handled male C57BL/6J mice also consumed less sucrose and had smaller lick cluster sizes in the voluntary sucrose consumption test compared to mice handled with their home tunnel, indicating that they were more anhedonic (a core symptom of depression). Similarly, Sensini et al. (2020) found that single-housed male C57BL/6NCrl mice who were tail handled five times per week demonstrated more depressive-like behavior in the forced swim test* and lower overall well-being in the burrowing test compared to tunnel-handled mice; these effects were not seen in females. Both sexes were more willing to interact with the handler (via cupped hands or hand holding a tunnel) before and after tunnel handling compared to tail handling, and defensive burying (an indicator of anxiety) was seen only in tail-handled mice. Davies et al. (2022) tested whether mouse behavior upon arrival at a research institution would differ depending on the handling method the mice had experienced at the breeding facility. From birth until shipping, a mix of male and female wildtype and mutant mice primarily of a C57BL/6 genetic background were handled either by tail, tunnel, or cupping during all routine husbandry. The receiving technician—who was blind to previous handling experience—scored mouse behaviors upon arrival at the research facility. They found that mice who had been tail handled were more reactive to the box opening, were more difficult to handle, and scored lower on a hand approach test.

While grabbing and lifting mice by the tail using one's hand is detrimental, doing so using forceps appears to be even worse. Mertens et al. (2019) found that handling single- and group-housed male C57BL/6NCrl mice by the tail using forceps resulted in stress-induced hyperthermia and lower quality nests compared to using a hand to pick them up by the tail or using a tunnel. Tunnel-handled mice had the lowest levels of anxiety in the light/dark box test. In group-housed mice, tail handling using forceps resulted in the most aggression after cage changing. Hull et al. (2022) found that breeding pairs of C57BL/6J mice who were tail handled with forceps averaged one pup less per litter, suffered more litter losses prior to weaning, and had a 20% higher risk of recurrent litter loss compared to breeding pairs who were tunnel handled. All mice were housed with one cotton Nestlet (this is not enough for mice to build a proper nest), but those who were tunnel handled were additionally housed with that tunnel. Therefore, it is impossible to say what proportion of the benefits were due to the handling method

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** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

versus the availability of additional insulation (tunnel) in the cage. In an identical study comparing tunnel to tail handling with forceps using different strains—BALB/cJ and CD1 IGS—no differences were found in breeding success (Hull et al., 2024).

Hurst and West (2010) demonstrated that restraining mice by the scruff or by the base of the tail for abdominal inspection after picking them up using a tunnel or cupping did not reverse the increased voluntary interaction with the handler seen with these two methods. Similarly, Roughan and Sevenoaks (2019) found that male and female group-housed BALB/cAnNCrl mice handled with their home tunnel interacted much more with the handler's hand compared with tail-handled mice, and that interaction remained higher even after the mice were subjected to tail tattooing or ear tagging and were otherwise found to be anxious and experiencing some pain. Using male and female pair-housed BALB/c mice, Henderson, Dani, et al. (2020) investigated whether the benefits of home-tunnel handling persisted after mice were subjected to repeated scruff restraint, IP injections, and anesthesia. Repeated scruff restraint reduced the amount of time all mice spent interacting with the handler's hand, but tunnel-handled mice nonetheless interacted more with the handler's hand than tail-handled mice. Tunnel-handled mice exhibited lower levels of anxiety in the elevated plus maze test and the open field tests, with no effect of repeated restraint on this measure. Tunnel-handled mice who experienced repeated IP injections or anesthesia interacted more with the handler the day after these procedures compared to tail-handled mice; they also showed lower anxiety in the elevated plus maze test. Similarly, Gouveia and Hurst (2019) found that pair-housed male and female C57BL/6JOlaHsd mice picked up with home tunnel or cupping showed substantially more voluntary interaction with the handler than those picked up by tail, both immediately before and after repeated scruff restraint, with tunnel-handled mice interacting the most. Pair-housed female BALB/cOlaHsd mice interacted more with the handler and showed lower anxiety in a modified open field test when they were handled via a tunnel rather than by the tail, even when tested immediately after repeated subcutaneous injection. These studies demonstrate that the benefits of non-aversive handling methods are not "undone" even if the mice experience something stressful immediately before or after handling.

Gouveia and Hurst (2019) also showed that four cage-changing sessions were enough for tunnel- and cup-handled mice to show lower anxiety in the elevated plus maze test compared to tail-handled mice, and for tunnel-handled mice to spend more time voluntarily interacting with the handling device compared to mice who were cup or tail handled. The addition of nine daily 2-second handling sessions increased voluntary interaction with a hand in cup-handled mice but not in tail-handled mice,

providing further evidence that tail handling is aversive. Thus, the method of handling used during routine husbandry can impact mouse welfare, even if it is relatively brief or infrequent; prolonged periods of tunnel handling are not necessary to obtain the welfare benefits of this method.

Tail handling remains common despite overwhelming evidence that this practice is aversive to mice, prompting some researchers to explore the barriers to switching to alternative handling techniques. Two years after the benefits of non-aversive handling methods were first published, Waters (2017) surveyed workshop attendants at the 2012 Institute of Animal Technology Congress in the United Kingdom and found that most respondents used tail handling for mice, citing time constraints as their main concern. To test whether time constraints were a valid concern, Waters (2017) conducted a pilot study that found, contrary to people's fears, that tunnel or cup handling did not take longer than tail handling during routine cage changing. In a different pilot study, A. Swan (2018) similarly found that tunnel handling during cage changing did not take longer than tail handling, once tunnel handling had been in place for 3 months and technicians became more familiar with the method. Conversely, Doerning et al. (2019) found that using forceps or a hand to pick up mice by the tail were the fastest methods of transferring them compared to use of tunnels or disposable cups, and recommend the use of tail handling with gloved hands as a minor refinement over forceps (however, this prioritizes speed over welfare). They also found that handling methods did not impact animal health reports during the study and that providing tunnels in the home cage did not inhibit technicians' ability to perform health checks—debunking another common concern. Thorpe (2020) ran a small pilot study, demonstrating that transgenic male mice generally enter handling tunnels faster over time; by the end of the 12-week study, time to enter the tunnel was reduced by 50% compared to week 1, and tunnel handling took 2.4 seconds longer per mouse, on average, than tail handling.

A 2019 survey of international mouse users (31% from the United Kingdom, with over two dozen other nations represented in smaller percentages) found that most respondents used either tail handling (35%) or a combination of tail handling and other methods (43%) to pick up mice (Henderson, Smulders, et al., 2020). Researchers—compared to animal care staff—were more likely to have never heard of non-aversive handling methods and to use tail handling. Respondents still expressed concerns that alternative handling methods took longer; they also perceived that tunnel handling was incompatible with other procedures, such as health checking or restraint. A subsequent survey of mouse users (79% of whom were from the United States) found that, despite positive attitudes toward refined handling methods,

only 10% of respondents reported using tunnel or cup handling exclusively (Young et al., 2023). Respondents estimated that the vast majority of mice in their facilities were handled by the tail as the default method. They viewed refined methods as beneficial for mouse welfare (88% of respondents) and science (77%) but expressed no strong intent to use them personally in the next year. The main perceived barriers to implementation of refined handling methods were (1) mice making it too difficult (e.g., they are too "jumpy"), (2) incompatibility with research or procedures, (3) took too much time, (4) insufficient materials, (5) lack of personnel cooperation or support, and (6) potential harm to personnel (e.g., getting bitten). Given the growing body of literature indicating that tunnel handling is both practical and beneficial, and its increasing widespread implementation at research institutions, several of these concerns might be addressed with additional personnel training.

A recent study documented the implementation of tunnel handling in a large mouse-breeding facility (Hohlbaum et al., 2024). Three animal technicians with more than 10 years of experience with tail handling received 90 minutes of theoretical training and 120 minutes of practical training in tunnel handling. Over the next 10 months, they used tail or tunnel handling with male and female mice of three strains (Hello Kitty, WNK 1, and NZW) during weekly cage changing, for up to 9 weeks starting when mice were weaned. Tunnel handling occurred with the animals' home-cage tunnel. Tail-handled mice of all strains defecated more during capture compared to tunnel-handled mice, indicating that tail handling was more stressful. Hello Kitty and WNK 1 mice hardly interacted with the handler throughout the study, but tunnel-handled NZW mice spent more time voluntarily interacting with the handler than tail-handled mice. Time to transfer was longer using the tunnel than the tail in Hello Kitty mice, who did not seem to habituate to either method. For WNK 1 mice, time to transfer was similar for the two handling methods throughout. In NZW mice, time to transfer via tunnel decreased over time until it was similar to that of tail-handled mice. The authors concluded that their strain-specific results underscore the need for researchers to publish about their own experiences using non-aversive handling methods with different strains. They also acknowledged that all three handlers self-reported that they were still learning the practice of tunnel handling at the beginning of the study, compared to tail handling, with which they were very comfortable.

As described above, many users continue to use tail handling, either because they lack access to tunnels or because of fears that tunnel handling would interfere with other procedures, such as health checks. Additionally, Moore and Wickert (2021) found that some mice do not like tunnel handling; they inferred this from mice who would refuse to enter a tunnel, aiming their nose away from it, or pushing against it.

However, the authors found that other alternatives to tail handling worked very well, such as using a clean wire lid for mice to walk onto or guiding mice into an inverted home-cage hut (Figure 4). They suggested that technicians should be familiar with several practical alternatives to tail handling and should be attentive to individual mice who may indicate which capture method they prefer. Indeed, a few experimental studies have shown that tunnels or cupping are not the only humane alternatives to tail handling. Using pair-housed male and female C57BL/6NRj mice, Sandgren et al. (2021) found that handling with either a small plastic ladder (which is standard cage enrichment at their facility) or a home tunnel resulted in more voluntary interaction with the handler and lower anxiety in the open field test compared to tail handling. In the elevated plus maze test, anxiety was lower in ladder-handled mice than in tail-handled mice, but higher than in tunnel-handled mice. The authors concluded that using a home-cage ladder is a refinement over tail handling, conferring similar benefits to tunnel handling. Similarly, Wallin (2023) found that, compared to tail-handled mice, male and female CBB mice (a cross between C57BL/6 and BALB/c) handled with upturned huts from their home cage exhibited less defecation (1 vs. 27 times), vocalization (0 vs. 18 times), and anxiety in the open field test, and more voluntary interaction with the handler. Finally, Bodnar et al. (2025) also found upturned huts to be a humane handling method for male and female mice of various strains: Mice handled with an upturned hut spent more time voluntarily interacting with the handling object than tunnel-handled or cupped mice, and there were no differences between these three non-aversive handling methods in the elevated plus maze test.

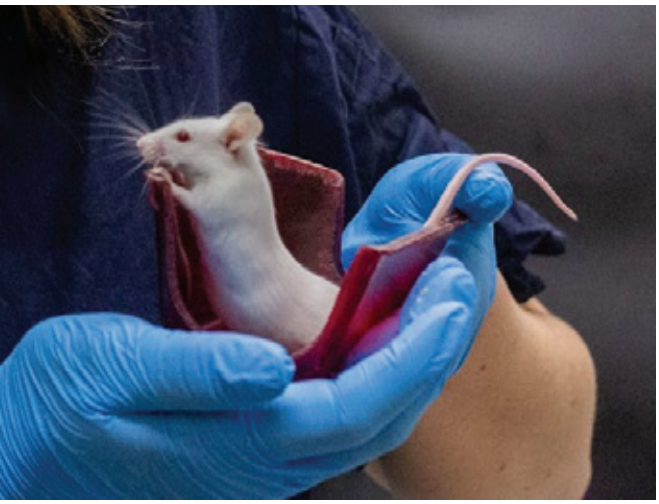

Figure 4: Inverted home cage huts are suitable for picking up and handling mice.

The researchers behind the 3Hs Initiative (see section on **Mice and Rats** above) developed a method to restrain mice for IP injection without holding onto the tail at any point. Mice are removed from their cage using cupping and placed on the handler's forearm. The free hand is placed over the mouse, with their head jutting out from between the thumb and index finger. The mouse is scruffed from this position, and the tail is never held. In a preliminary study, they compared this novel method (cup handling onto the forearm and scruffing from this position) to the conventional method (cup handling onto the forearm, restraining by the tail, and scruffing from this position) and found that mice handled with the novel method displayed significantly less struggling, urination, defecation, and aversion on release (Bartlett et al., 2022). In a formal study by researchers from the same research group, C57BL/6 and ICR (CD1) mice of both sexes showed less struggling, vocalization, and aversion on release when handled with the novel method compared to the conventional method (Davies et al., 2022). There were no differences between groups in serum corticosterone or anxiety in a variety of behavioral tests. Thus, these studies would suggest that holding onto the tail at any point—not just during capture and lifting—may be aversive to mice.

The specific method used to scruff a mouse can also have profound effects on mouse physiology, behavior, and welfare. Labitt et al. (2021) demonstrated that standard scruffing restraint with two fingers (stretching the skin to create a longitudinal fold across the neck and immobilizing the forelimbs) of conscious mice results in abnormal cardiovascular parameters in male and female mice of four strains (BALB/cJ, C57BL/6J, DBA/2J, and FVB/nJ). Compared to restraint with *three* fingers (pulling the skin to create a transverse fold; Figure 5) or light restraint (gently pressing down on the mouse and leaving the head free to move), standard two-finger scruffing restraint caused severe arrythmias and an average of 31% reduction in heart rate, with a maximal reduction of 79%; bradycardia (abnormal heart rate) persisted for several minutes after release from restraint. Some mice restrained in this manner also demonstrated gasping behavior. The authors suggest avoiding the use of two-finger scruffing restraint due to profound cardiovascular impacts.

Placing animals into restraint tubes should also be avoided unless, perhaps, the animals are first habituated to the procedure using positive reinforcement (see section on **Mice and Rats** above and Sikora et al. (2016) below). Using single-housed male CD1 mice, Redaelli et al. (2021) showed that one-time restraint in a small Plexiglas tube under bright light (400–500 lux) for 15 minutes—as is commonly done for intravenous substance injections—caused physiological and behavioral changes consistent with moderately severe stress (reductions in peripheral body temperature, increased grooming, and reduced walking and rearing in the home cage 24 hours after such restraint).

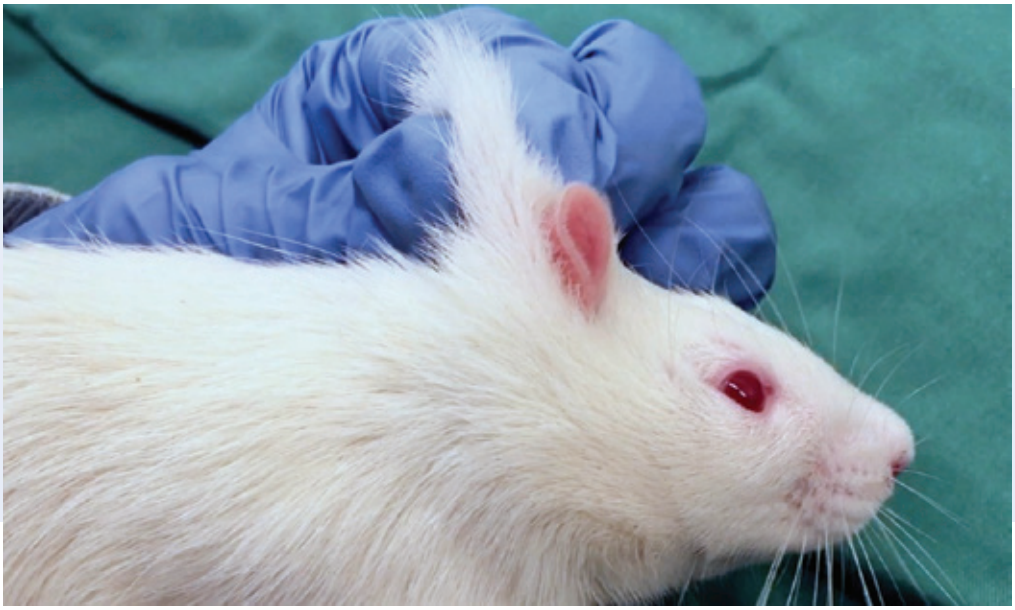

Figure 5: When scruffing mice, a transverse skin fold eliminates pressure on the throat area and thus avoids profound cardiovascular impacts (Labitt et al., 2021).

HABITUATION AND TRAINING

Habituation to handling, restraint, and experimental procedures can decrease mouse discomfort during these procedures. For example, Swan et al. (2023) divided socially housed male and female CD1 mice into two groups: The control group was subjected to a standard laboratory protocol, which was tail handling during weekly cage changes and physical examinations, while the treatment group was cup handled during these weekly routine procedures, in addition to receiving training (five times per week for 5 weeks, 8–10 seconds per session) during which they were placed on a Vetbed and their skin was lifted and pinched to simulate a subcutaneous injection. Using the Mouse Grimace Scale to assess pain or discomfort (see NC3Rs resource on grimace scales: https://perma.cc/E2TM-H325), the authors found that both sexes from the treatment group had lower ear scores during subcutaneous injection and tail vein blood sampling, indicating that these mice experienced less discomfort during both procedures. Unfortunately, because handling method was confounded with habituation to the injection procedure, it is impossible to determine the relative contribution of handling method versus habituation to injection on the results.

Habituation to handling and restraint may also help reduce stress-induced weight loss related to oral gavage. Using group-housed female C57BL/6JHsdOla mice, Kärrberg et

Figure 6: Mice can be trained to consume substances from a syringe voluntarily while unrestrained.

al. (2016) compared mice across three treatments: undisturbed for 6 days, subjected to 10-second restraint over 6 days, or subjected to sham gavage with a dry needle over 6 days. All mice were then subjected to twice daily oral gavage for 8 days. Mice in the undisturbed treatment lost significantly more weight over the 8-day experimental phase than mice in the other two treatments. There was no difference between mice in the restraint versus the sham gavage treatments; therefore, habituation to restraint alone appears to be a refinement over habituation to sham gavage.

Gavage is invasive and prone to complications in mice; animals should be trained to voluntarily consume substances instead. In a small descriptive study, Krall et al. (2023) found that mice were more willing to consume a substance from a syringe when they were in a playpen than when they were handheld next to their home cage. Briefly, the authors first habituated mice to cupping over 1 week and then trained them to feed from a syringe for another week. Habituation to cupping consisted of placing a hand in the cage with the palm up to allow the mouse to explore it, covering the mouse with the other hand to simulate a burrow, and scooping the mouse with both hands. Habituation and training to syringe feeding consisted of coating the tip of a syringe with Nutella (high-value reward) and placing it on the cage floor, placing a drop of Nesquik (medium-value reward that would be used as a vehicle for the drug) on the nape of the mouse's neck, which would be tasted during grooming, and ejecting Nesquik from the syringe, drop by drop, to allow the mouse to consume it voluntarily while unrestrained (Figure 6). When mice were trained to drink from the syringe while being cupped next to their home cage, only one mouse drank the entire

contents of the syringe over the course of 10 sessions. In contrast, when mice were presented with the syringe while they were free to move inside the playpen, all mice drank the entire contents on the first session. However, because handling method and location were confounded (handheld next to the home cage versus free moving in the playpen), it is impossible to determine the relative contribution of these two factors.

Dickmann et al. (2022) demonstrated how positive reinforcement training can be used to increase mouse "compliance" and walking speed in the catwalk test (a measure of mouse locomotion and gait that requires mice to move at a consistent pace), rather than using aversive stimuli (e.g., air puffs) to force mice to complete the test. Using a clicker and food reward, C57BL/6 mice of both sexes were trained over 11 days to participate in a catwalk test. Trained mice ran faster during the test, required fewer runs to complete data collection, and demonstrated lower anxiety in the elevated plus maze tests and open field tests.

Finally, refining the methods used to train *humans* how to handle and perform procedures on mice can benefit both humans and animals. Benjamin (2024) described how the use of a scruffing restraint device (ScruffGuard) has helped new staff gain confidence and familiarity with the animals, use fewer animals to reach training goals, and reduce injuries to both humans and animals. This restraint device is made of durable cardboard and comes in various sizes; it is designed to be placed over a mouse standing on a flat surface. The loose skin can be pulled through the opening at the top, removing the possibility of being bitten. However, in light of the results described above (Labitt et al., 2021) showing the risks associated with a longitudinal skin fold, the tightness of the hold and the breath rate of the mouse should be carefully monitored if this device is used.

Rats

HANDLING AND RESTRAINT

Taniuchi et al. (2019) found that handling is stressful to male Wistar rats, even after they were habituated to handling for 1 minute per day for 10 days. The authors based their conclusion on the finding that rats who were "carefully" moved manually by the experimenter from a maze to the home cage performed less well on the maze learning tasks compared to either rats who received a food reward after training or rats who could return to their home cage on their own through a tunnel. This indicated that handling had a negative impact because it impaired learning, but the article did not specify what handling method was used—and many methods exist.

In a survey of self-reported handling methods and outcomes from rat users, Burn et al. (2023) found differences in the prevalence of various methods and their perceived effects on rats. One-handed dorsal lifting (aka "shoulder saddle" or "standard thoracic hold"), which consists of encircling the torso from behind at shoulder level and grasping the rat around the thorax, was the most commonly reported method (77%). This approach was often how respondents were taught to lift rats and was perceived to be comfortable for the rat; however, respondents also reported that dorsally lifted rats were significantly less likely to approach their hand than rats who were handled using methods such as tunnel handling, cup handling, or handling with support on the rat's chest and bottom. Cup handling and handling with chest and bottom support were perceived to be associated with more positive behaviors. Lifting rats by the base of the tail was reported in 39% of workplaces and had the most reported concerns about animal welfare due to signs of rat stress (e.g., defecation, less likely to approach handler).

An experimental study using male and female single-housed Crl:CDSC rats compared rats' responses (vocalizations, hypersensitivity to touch, activity in the open field test) to lifting with a tunnel or lifting using the one-handed dorsal technique while wearing a nitrile glove, wearing a protective bite glove, or holding a soft paper towel (Wypall). In males, dorsal lifting with a paper towel was least stressful, dorsal lifting with a nitrile glove was intermediate, and dorsal lifting with a protective glove or lifting with a tunnel were most stressful. In females, differences between handling methods were less pronounced, but open field activity suggested they were least anxious when handled with a tunnel and most anxious when handled with a paper towel or protective glove, compared to handling with a standard nitrile glove (Kylie et al., 2024). The only consistency between males and females was that dorsal lifting with the protective bite glove was the most aversive handling method.

Full restraint is aversive to rats—sometimes more so than the procedure they are being restrained for. For example, Sikora et al. (2016) found that habituation to restraint in a plastic restrainer does not help to reduce stress in male Sprague Dawley rats: The animals' arterial blood pressure and heart rate, which were continuously recorded via implanted telemetry, increased significantly when they were placed inside a restraint tube for an hour, whether the animals had been habituated to this procedure for 2 weeks (7 habituation sessions), 4 weeks (14 habituation sessions), or not at all. Moreover, repeated restraint altered diurnal variation in arterial blood pressure. The authors concluded that rats show no hemodynamic adaptation to repeated restraint and that physiological measurements using restrained rats are confounded by stress-induced acute and chronic changes in arterial blood pressure.

Gentler restraint is recommended whenever possible. Working with Wistar and Lister hooded rats, Stuart and Robinson (2015) performed IP injections using either conventional scruffing restraint (pulling the skin back to restrict movement of the head and arms) or a modified method in which the animal's hind legs are rested against the researcher's torso and the animal's head and front paws are free to move (Figure 7). The modified method resulted in lower corticosterone levels and less struggling, vocalization, and defecation during injection. Moreover, rats who were conventionally scruffed demonstrated a more negative emotional state in a behavioral test of affective bias.

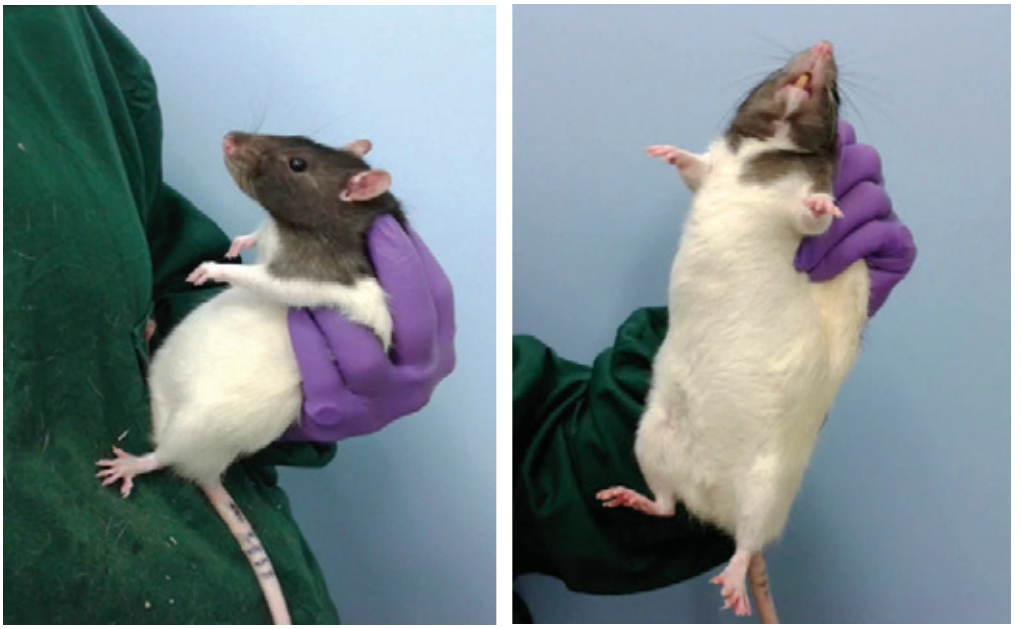

Figure 7: In Stuart and Robinson (2015), a modified restraint method in which the rat's hind legs are rested against the researcher's torso and the head and front paws are free to move (left) resulted in lower stress and anxiety compared to the standard scruffing method (right).

As another restraint refinement, Ludwig (2020) suggests using a sewn cloth pocket with an adjustable rear strap that secures around the hind legs (Figure 8). The author noted that most rats will willingly enter the dark pocket and that it is more efficient than using a folded towel for restraint. Another creative restraint method is to use a sock, which can be turned into a modified "thunder jacket." Byrd et al. (2018) outlined a protocol for this restraint method using sterilized 100% cotton socks with a small hole (< 0.5 in.) cut into the toe section for the rat's head to fit through. Rats are first

Figure 8: A sewn cloth pocket with an adjustable rear strap that secures around a rat's hind legs is more efficient than using a folded towel for restraint (Ludwig, 2020).

habituated to human touch, then to being covered with a hand towel, and finally to being wrapped with the sock. The habituation procedure takes 2–7 days. Once rats are accustomed to the process, blood collection or compound administration could be performed with the rats restrained comfortably in a sock. The authors noted anecdotally that this helped the animals remain calmer, while the staff were more confident and efficient when performing procedures requiring restraint.

Blood pressure can be measured noninvasively using the tail-cuff method, but this method nonetheless requires confinement in a restraint chamber, which may cause stress. Lipták et al. (2017) describe a modified tail-cuff method to measure blood pressure in lightly restrained rats. Their method involves covering the rats with a sheet on a procedure table, allowing them to explore, and gently holding them while applying the cuff to the base of the tail; if the rat struggles, they are released. When comparing the two methods, the authors found that rats restrained in a chamber demonstrated more struggling, vocalization, and defecation. Blood pressure values tended to be lower for rats using the modified restraint method, indicating lower stress and likely a more accurate representation of baseline blood pressure. Avoiding the use of full restraint whenever possible is a very simple refinement to reduce stress and improve welfare.

HABITUATION

There is a large body of literature promoting the use of "tickling" as a beneficial method for humans to interact with rats housed in laboratories. Tickling is a form of heterospecific play in which humans make light, brisk, and vigorous movements with their fingertips on a rat's neck and abdomen to imitate juvenile rat rough-and-tumble play (Figure 9). While tickling may not be the equivalent of conspecific play, rats still enjoy it (Burke et al., 2022). Many rats will vocalize in the 50-kHz range during tickling, and this vocalization has been likened to human laughter (Panksepp, 2007). Such playful interaction can better habituate rats to humans and help build and strengthen a positive relationship. In a systematic review of 32 articles on rat tickling, LaFollette et al. (2017) concluded that rats who are tickled emit more 50-kHz vocalizations, show improved human approach behavior, are more easily handled, and exhibit lower stress and anxiety; these effects are most pronounced in juvenile, single-housed rats.

Cloutier et al. (2012) assessed responses of male Sprague Dawley rats to four handling treatments: once weekly during cage changing, daily exposure to a passive hand for 2 minutes, daily tickling for 2 minutes, or restrained on their back for 2 minutes* (to

Figure 9: "Tickling" is a form of playful interaction that can help build and strengthen positive relationships with rats. A bat detector can be used to hear rats' vocalizations during tickling in real time to confirm whether individual rats are enjoying the interaction, especially when it involves pinning (right).

** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

mimic pinning by a dominant rat). The handling treatments did not impact rats' ease of handling during weighing, behavior in an elevated plus maze test, or behavior in a cat-odor test of anxiety. However, the tickling treatment was the most effective method for reducing rats' fear of humans in a human approach test compared to rats who were minimally handled. Rats in the restraint treatment still approached a handler as often as rats in the tickling treatment, but they were less likely to rear or solicit play (e.g., nibble the hand) during these interactions. In another experiment, Cloutier et al. (2013) investigated the effects of daily tickling (2 minutes per day for 3 weeks) compared to minimal handling (weekly during cage changing) on juvenile male Sprague Dawley rats who were housed singly or in groups. Tickled, single-housed rats exhibited lower anxiety in the open field test and produced more 50-kHz vocalizations in anticipation of tickling compared to minimally handled rats. There were no differences in group-housed rats. In one of the only studies that looked at tickling in female rats, Tivey et al. (2022) confirmed that Wistar rats of both sexes react similarly to tickling. The authors assessed 50-kHz vocalizations during tickle-induced play behaviors (hopping and darting) rather than during actual hand-to-rat contact; they found that both sexes laughed before and during these behaviors and in anticipation of the human hand approaching them.

Tickling can also reduce the negative impact of painful procedures or stressful experiences. Cloutier et al. (2014) demonstrated that 2 minutes of playful tickling of male Sprague Dawley rats before and after repeated IP injections (compared to exposure to a passive hand before and after the injections) resulted in more 50-kHz vocalizations before and after injection, fewer audible vocalizations (indicative of pain or discomfort) during injection, and faster injection procedures. These findings were stronger in rats who had been previously tickled as juveniles, rather than tickled only as adults. In a similar study using juvenile male Sprague Dawley rats, Cloutier et al. (2015) demonstrated that brief tickling before a mildly aversive procedure, such as restraint and IP injection, has a carryover effect during the procedure; as such, tickling is more effective when applied before rather than after a procedure. Finally, a research group studying the effects of socially induced positive emotions on various adverse life events found that single-housed juvenile male Fischer rats who were tickled for 2 minutes per day, 5 days per week for 2 or 4 weeks before fear conditioning had less fear-induced freezing, better learning ability in the Morris water maze, and lower plasma adrenaline and noradrenaline (Hori et al., 2013, 2014).

When implementing rat tickling, there are several variables to consider. For example, LaFollette et al. (2018) set out to determine the minimum effective tickling "dosage." Testing three tickling frequencies (1, 3, or 5 times) and durations (15, 30, or 60

seconds) in pair-housed male and female Long-Evans rats, they determined that tickling for 15 seconds at a time for 3 days was the most effective and efficient dosage. Rats tickled three times per week produced the most 50-kHz vocalizations before and during tickling, and they played more and showed more anticipatory activity in the hour prior to tickling when compared to rats who were tickled only once per week. There were no differences in any outcomes based on tickling duration, nor were there any effects on rats' approach behavior, their fecal corticosterone levels, or the duration of injection procedures. This demonstrates that rats can be habituated to tickling with a relatively small investment of time. In another study, LaFollette, Swan, et al. (2019) found that light intensity and cage color can affect rat emotional responses to tickling. Indeed, juvenile male Long-Evans and CD rats (one rat of each strain per cage) emitted more positive 50-kHz vocalizations when housed and tickled under intermediate lighting conditions (red cages under 200 lux and clear cages under 25 lux) compared to dark (red cages under 25 lux) or bright (clear cages under 200 lux) conditions. Finally, Lomax et al. (2024) determined that pregnant dams can safely be tickled without it affecting dam health or fetus implantation. Dams were tickled a few times 4 to 20 days *post coitum*. Also, extra care was taken when flipping the rats onto their backs, and tickling was performed a little bit higher on the belly than normal.

A survey of laboratory personnel found that, despite strong scientific evidence that rat tickling is an effective way to improve rat welfare, 55% of participants had never used this technique; one self-reported barrier to implementation was participants' lack of confidence in their ability to do it correctly (LaFollette, Cloutier, et al., 2019). Because standard hands-on training is not easily accessible, researchers sought to determine whether online training could be an effective alternative. The study determined that a combination of online and hands-on training was most effective at improving implementation of tickling, although online-only training still resulted in improved knowledge, self-efficacy, and implementation of rat tickling (LaFollette et al., 2020). The online training course is available for free at https://perma.cc/T7AR-KHZ5. In addition, a video demonstration of a standardized rat tickling procedure is available in the *Journal of Visualized Experiments* (Cloutier et al., 2018), and various training resources are available on the 3RsC and NC3Rs websites.

An important caveat with rat tickling is that at a *population* level, tickling induces a positive affective state, but *individual* rats vary in how much they enjoy the experience. For example, Rygula et al. (2012) found that only about half of their group-housed, male Sprague Dawley rats laughed when they were tickled, and only this half were in a more positive affective state after tickling compared to rats who were not tickled. The other half laughed little or not at all during tickling and were not in a more

positive affective state after tickling. Similarly, using pair-housed, male Lister hooded rats, Hinchcliffe et al. (2020) found that not all rats like to be tickled, and that 50-kHz ultrasonic vocalizations (i.e., laughing) are an effective real-time indicator of whether individual rats are enjoying the experience. (An inexpensive bat detector can be used to allow human ears to detect 50-kHz vocalizations in real time.) They advised handlers to take care to identify which individuals actually enjoy being tickled.

It has been suggested that pinning rats on their back during tickling is the aspect of tickling that some rats dislike. Traditional tickling techniques rely heavily on pinning rats on their back to tickle their abdomen, because rats who do laugh will laugh the most in this position. An international group of scientists proposed that current tickling methods could be improved to make tickling more inclusive of different rat personalities (Bombail et al., 2021). When rats play together, some occasionally *choose* to roll onto their backs to be pinned by their play companion. However, during tickling, pinning is used much more often than during rat social play, and *a human* decides when a rat should be grasped and flipped onto their back. The scientists suggest modifying current tickling protocols to incorporate more aspects of rat play (such as chasing, sparring, and wrestling), reduce the amount of pinning, and pay attention to individual rats' responses during each session. The authors believe that the result "would be a more inclusive tickling method that is more playlike and likely to be a pleasant experience for more rats, including individuals [who] do not enjoy being pinned."

More traditional methods of habituating rats to human interaction involve so-called "gentling" protocols, which are particularly effective at reducing general and human-directed fear and anxiety. In one experiment, Costa et al. (2012) found that single-housed male Sprague Dawley rats who were placed on the experimenter's lap or a table and gently stroked on the neck and back for 5 minutes five times per week for 6 weeks (total time investment: 150 minutes) as adolescents demonstrated lower anxiety in the elevated plus maze test and better learning and memory in the retention test compared to rats who were handled only during cage changing. Similarly, group-housed juvenile female Wistar rats who were gently lifted twice and touched with a bare hand on their body and tail for 10 minutes daily for 2 weeks (total time investment: 140 minutes) exhibited fewer anxiety-related behaviors during various interactions with an experimenter (e.g., being picked up or touched) compared to rats who were handled only during cage changing (Schneider et al., 2016). The authors noted that this 2-week gentling program had less of a long-term impact compared to a similar program that lasted 4 weeks (total time investment: 280 minutes) and included hand feeding (Maurer et al., 2008), but both were effective at reducing rats' fear of humans. In another example, adolescent pair-housed male

Lewis rats who were gently stroked on the back with a cotton glove (to reduce friction compared to a standard nitrile/latex glove) while sitting on the experimenter's lap for 5 minutes every other day for 4 or 8 weeks (total time investment: 70 or 140 minutes) had higher activation of oxytocin neurons (these are involved in positive affiliative behaviors) and also showed more affiliative behavior toward humans (more time in proximity to and following the experimenter's hand) compared to rats who were not stroked (Okabe et al., 2020). Adolescent group-housed male Wistar rats who were gently touched on the face, neck, and back for 20 minutes five times per week for 12 weeks (total time investment: 1,200 minutes) demonstrated lower anxiety in the open field test and lower depressive behavior in the forced swim test* compared to rats who were handled only at cage changing (Song et al., 2021). Finally, Costa et al. (2020) found that tactile stimulation could be an effective intervention in a rat model of chronic stress. Single-housed male Sprague Dawley rats were subjected to a mild chronic unpredictable stress protocol for 3 weeks, but some were also gently handled 5 days per week for 3 weeks during that stress period. Rats who were handled had lower corticosterone levels, less depressive behavior in a forced swim test* and a sucrose preference test, and better learning ability in a Y-maze.

Rats who are used in institutional training workshops for new users also benefit from prior habituation to handling. Naïve rats may react fearfully to handling and restraint, putting everyone's welfare and safety at risk. To reduce stress associated with a rat handling training workshop, Low et al. (2019) habituated rats to handling and restraint three times per day over the course of 3 days before the training workshop. Rats were first stroked, patted, scratched, or tickled around the nape and head; then, they were lifted by scooping one hand under their chest while the rump was supported in the other hand. Finally, rats were gently and swiftly turned onto their back to have their belly tickled before experiencing a common manual restraint procedure. The authors observed that this process made rats calmer during handling sessions, with zero bite incidents occurring since this habituation protocol was implemented.

Other Rodents

Chinchillas are prolific jumpers and climbers, so practice with handling (as well as patience) is crucial to prevent escapes, injuries, and "fur slip"—a defense mechanism that allows them to release large patches of fur in an attempt to avoid capture

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** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

(LaFleur & Williams-Fritze, 2020). Chinchillas should not be handled by the scruff or by the base of their tail alone. Their body should be supported with one hand, while the other hand may hold the base of the tail. Once habituated, chinchillas will readily accept treats from the hands of staff, often running up to the front of the cage in anticipation (Figure 10).

Baskir et al. (2020) demonstrated that chinchillas can learn to use visual cues to anticipate and prepare for upcoming events. In their study using male and female zoo-housed chinchillas, a handler always wore green latex gloves for husbandry activities such as cleaning (which can be stressful), and brown leather gloves for handling sessions outside of the animals' home enclosures. Later, when cleaning gloves were used for handling sessions, chinchillas were significantly more aroused (e.g., alert posture). This suggests that they had developed expectations based on the cue of the gloves and used these cues to prepare for predictable stressful events and adjust their behavior accordingly. Therefore, reliably signaling mildly aversive events could be beneficial for animal coping.

De Lima Rocha et al. (2017) investigated the effects of habituation to handling in **guinea pigs**. Animals were allocated to one of three treatment groups: no contact with the experimenter, brief handling during cage changing, and daily habituation to the experimenter (gentle handling and stroking). After 3 days of habituation, the guinea pigs no longer showed signs of distress and interacted with the experimenter. Habituation to contact with the handler also resulted in longer latency for animals to reach tonic immobility (a rigid fear response to predator interactions) when inverted in a restraint trough.* In a second test, they found a social learning effect of habituation: Unhabituated guinea pigs housed with habituated guinea pigs also showed decreased tonic immobility during inverted restraint, indicating a possible reduction of fear due to their exposure to habituated guinea pigs.

Naked mole rats (NMRs) may lack a fur coat, but they are covered with vibrissae that make them very sensitive to touch. Gentle lifting or cupping are recommended for handling. NMRs should never be picked up by their tails, which are also covered with vibrissae and play an important role in their sensory system (Ragland et al., 2022; Smith & Buffenstein, 2021). They are timid and startle easily, but gradually become habituated to the lab after 3 to 6 months of fixed feeding and cleaning times (Yu et al., 2017).

** This methodology is deemed to cause undue harm or suffering in addressing the research question.*

Damaraland mole rats who are handled regularly habituate fairly quickly and can be picked up by gently placing a hand around their abdomen. However, handlers must use caution when scruffing the animals, who may bite if they become stressed; to avoid being bitten, a handler can place a thumb under the mole rat's chin while the rest of the hand holds them around the body (Buffenstein et al., 2024).

Figure 10: Once chinchillas are habituated to humans, they will readily accept treats from the hands of staff, often running up to the front of the cage in anticipation.

Rabbits

HANDLING AND RESTRAINT

Most rabbits do not like to be lifted, held, or carried—they are much more comfortable on a solid surface near or on the ground (Bradbury, 2015; Buseth & Saunders, 2015). If rabbits need to be moved, they can be encouraged to enter a carrier or box on their own to reduce stress and give them back some control (A. G. Bradbury & Dickens, 2016; see also chapter on Transportation).

When handling is inevitable, a variety of methods are commonly used, but not all are welfare friendly. A survey of UK and Irish individuals who either owned, bred, or worked with rabbits in veterinary or laboratory settings revealed that laboratory workers differed from other groups in their handling methods (Oxley et al., 2019). For respondents overall, the most common handling method was to hold the rabbit upright against one's chest, with one hand supporting the rabbit's rear and the other supporting the back. For laboratory workers, however, the most common handling method was to rest the rabbit horizontally on the forearm with head tucked below the armpit. Only 15% of respondents overall handled rabbits by scruffing the skin of the neck while supporting the rear, while 75% of laboratory workers did so. Respondents who did not use scruffing described this method as "distressing," "painful," and "uncaring." Inverting rabbits on their back to induce "tonic immobility" or "trancing" was used by about half of the respondents, including lab workers. Those who did not use this method described it as "dangerous" and "wrong." In a literature review, A. G. Bradbury and Dickens (2016) indicated that, while it was traditionally thought that tonic immobility was induced by deep relaxation, the opposite has been found to be true: After being inverted, rabbits show significantly elevated respiration, heart rate, and plasma corticosterone, as well as behavioral changes indicative of fear. Indeed, there is now strong evidence that tonic immobility is a survival strategy following intense emotional stress. Giannico et al. (2014) showed that male and female New Zealand white rabbits exhibited many cardiologic changes the moment the rabbits were scruffed (which they say "somewhat emulates the subjugation produced by a predator's jaw"), followed by tonic immobility when they were placed in lateral recumbency (holding rabbits on their side). The authors concluded that their results reinforced the theory that tonic immobility was induced by fear responses in rabbits. Many organizations dedicated to rabbit care and welfare, such as the Rabbit Welfare Association and Fund, the RSPCA, and Rabbit.Org Foundation recommend against inverting rabbits to induce tonic immobility.

Cruden et al. (2012) described anecdotal observations that changing handling methods from day to day can cause the rabbits to display signs of stress, such as struggling and

foot stamping. They suggest that using one handling method consistently and giving rabbits time to settle before beginning a health inspection may keep them calmer.

HABITUATION

Working with male and female pet rabbits of various breeds, Unwin et al. (2020) tested the effects of Pet Remedy, a natural product containing valerian and marketed as an anxiolytic for companion animals. Exposure to Pet Remedy led to decreased heart rates during handling and increased positive behaviors (e.g., sniffing the environment, approaching the handler) in a novel environment, compared to a placebo. The authors suggest that Pet Remedy may be helpful to calm rabbits in acutely stressful scenarios, such as a physical exam by a novel handler. However, they also noted that the heart rates of all rabbits in their study were still much higher than normal, and the majority of rabbits showed aversion to human approach, indicating that handling was acutely stressful. They suggest that herbal interventions are insufficient, and handlers should employ additional efforts to reduce rabbits' fear of handling, including early socialization and habituation with human handlers, counter-conditioning with something positive such as food, and using low-stress techniques such as handling them on a surface rather than lifting them in the air.

Rabbits can be habituated to humans using relatively simple (and pleasant!) methods that require little time investment. For example, scientists at a facility that breeds rabbits developed a rabbit-human habituation program to reduce handling-induced stress in the animals (Esparza et al., 2024). The habituation program consists of three stages. In the *Imprinting Stage*, rabbits receive a daily petting session lasting 10 seconds in their nest box from birth to 5 weeks of age. In the *Reinforcement Stage*, rabbits receive three clinical exams with petting on weeks 6, 10, and 12. In the *Habituation Stage*, rabbits receive individual weekly petting from the age of 13 to 19 weeks. To test the efficacy of this habituation program, one of the company's clients evaluated clinical signs of stress in the rabbits during the acclimatization period at the new facility using a double blinded randomization study. Rabbits came from four different breeding rooms, only one of which implemented the habituation program. Rabbits were evaluated before and after the implementation of the habituation program. There was no change in stress scores of the rabbits from the three rooms that hadn't received habituation training. In rabbits from the room in which the habituation program was implemented, however, there was a significant reduction in overall stress scores: from 11% before implementation to 5% after implementation. Notably, none of the habituated rabbits received the highest stress score on any one of the clinical signs evaluated, and some signs—such as biting, vocalization, and need for critical care diet—were absent altogether in these rabbits.

In a very different setting—a new type of agroecology system where farmed rabbits have access to pasture with apple trees they help to fertilize as they graze—rabbits have also been found to benefit from positive habituation to humans (Fetiveau et al., 2024). The rabbits live in outdoor paddocks with wooden shelters. Half of the rabbits received minimal human contact that consisted of brief daily visual checks and weekly delivery of food, water, and health assessments, during which they were scruffed, with hands supporting their hind legs, for weighing. The other half of the rabbits received the same husbandry, except that rather than scruffing, they were grasped with both hands, one supporting the chest and the other wrapped around the pelvis. In addition, the experimenter spent approximately 10 minutes per day for 16 days sitting in the paddock with the gently handled group, offering rabbits food pellets made of timothy hay and carrots, and stroking individual rabbits and speaking to them in a soft voice as they ate. After this habituation period, an experimenter entered and stood in the paddock and then approached rabbits individually, attempting to stroke them. Rabbits who were handled gently and habituated spent more time near the experimenter and accepted more strokes.

With regard to handling and restraint, repeated daily exposure can help reduce rabbits' resistance to these procedures when they are applied during experiments. Over the course of 3 weeks, for a total of 14 sessions lasting 4 minutes each, single-housed adult female New Zealand white rabbits were removed from their cage, placed on a plastic cart covered with a nonslip mat, stroked gently between the eyes, and palpated slowly and with moderate pressure over the body. Then, they were scruffed and lifted into a plastic box, where they were held still against the side of the box while a gloved finger was placed on the artery of one ear to simulate the tactile contact used in blood collection. Treats were provided upon return to the home cage to help build a positive association with these procedures. When rabbits were subjected to this handling and restraint protocol 10, 14, and 21 days later, they were less resistant and less likely to flinch in response to the initial touch from the handler compared to rabbits who had only been handled during routine husbandry (Swennes et al., 2011). While such a habituation protocol is likely beneficial in terms of minimizing the risk of stress-related errors and improving scientific outcomes because the animals are calmer during data collection, the lifting method could be more appropriate and the habituation procedure more gradual so that it, too, is less stressful to the animals.

KEY TAKEAWAYS: HUMAN-ANIMAL INTERACTION

- All **rodents** and **rabbits** benefit from habituation and desensitization to human presence, handling, restraint, and other required laboratory procedures, particularly if those experiences are paired with rewards. Habituation, desensitization, and positive reinforcement training can decrease animals' fear and anxiety—making them easier to handle, improving their welfare, speeding up data collection, and improving data quality.

- Socializing and habituating **rodents** and **rabbits** to experimental scenarios prior to beginning a study can be achieved through daily gentle handling and stroking, exposure to relevant equipment such as restraint tunnels, provision of rewards, and getting animals accustomed to being touched in areas of the body from which samples might be taken during a study (e.g., their tail or hind limbs) in the days and weeks leading up to the procedure(s). Providing animals with reliable cues prior to procedures may also help them cope more effectively.

- Tail handling is very aversive to **mice** and should be replaced with tunnel handling, cupping, or handling with a familiar home-cage object (e.g., cage lid, ladder, or upturned hut). Handling a mouse by the tail for as little as 2 seconds results in avoidance of the handler. There is evidence that tunnel handling is the most impactful handling refinement, even if only performed infrequently for short durations (e.g., at cage changing), and its benefits persist even if mice are subjected to aversive procedures, such as restraint and injections, immediately after handling. Tunnel handling may initially take longer for inexperienced handlers, but this time is reduced as handlers and mice become more familiar with the procedure. **Rats** may also benefit from tunnel handling, cupping, or handling with support on the rat's chest and bottom.

- Many **rats** enjoy being tickled. Tickling has several welfare benefits and can be used to establish a positive human-animal relationship between rats and handlers. Rats can be habituated to tickling with a small investment of time; positive effects have been demonstrated with as little as three 15-second sessions. Not all rats respond in the same way to tickling, however, so handlers should take care to monitor and respond to individual rats' behavior, particularly when it comes to pinning the rats on their back during tickling bouts. Tickling rats before a mildly aversive procedure has carry-over effects to the procedure and may reduce rats' negative reaction to the procedure.

- → Full restraint is acutely stressful for **rats** and should be avoided and replaced with refined, less restrictive methods whenever possible.
- → Handling is acutely stressful for **rabbits**, making habituation and positive reinforcement particularly important when handling cannot be avoided. Pet Remedy (a natural product containing valerian) may be helpful to calm rabbits in acutely stressful scenarios but is insufficient to mitigate fear alone. Whenever possible, rabbits should be allowed to remain on a solid surface near or on the ground; lifting, holding, and carrying rabbits by hand should be avoided.
- → Inducing tonic immobility in **rabbits** by inverting them on their back induces intense emotional stress and should be avoided.

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Colony Management

What Is It, And Why Does It Matter?

The term “colony management” refers to husbandry practices related to the keeping and maintenance of large numbers of animals. While the list of colony management practices is long, this chapter focuses on practices that broadly relate to cleaning and hygiene, individual identification methods, and genotyping. When large numbers of animals are kept in close proximity, disease can spread quickly among the colony. Therefore, cleanliness and hygiene are important aspects of animal welfare, because animals who become ill are likely to experience negative physical and psychological functioning. Identification of individual animals can help ensure they receive appropriate care, such as health monitoring and medical treatment. Individual identification is also often required for research purposes.

That said, husbandry practices that promote hygienic conditions, reduce biohazard risk, and allow for individual identification can also cause stress to the animals. Health checks and cage changes require disturbing the animals and modifying or removing elements of the environment that are familiar to them, especially scents. Identification methods may require handling or restraint, cause pain, or risk infections. In other words, there is a welfare trade-off between maintaining a safe and hygienic environment for the animals and disturbing them in their safe space to perform the required husbandry procedures. The key is striking a balance that keeps animals healthy while minimizing disruptions.

Summaries of Current Refinement Research

Mice and Rats

CAGE CHANGING AND ROUTINE HUSBANDRY

Prolonged exposure to ammonia gas from urine-soaked bedding causes irritation that may lead to various pathologies in the rodent respiratory tract (Ferrecchia et al., 2014; Vogelweid et al., 2011). The commonly accepted airborne ammonia limit

is 25 parts per million (ppm), but it is important to recognize that this limit is based on parameters set for humans over a workday—not for rodents who are exposed to ammonia 24/7 (Eskandarani et al., 2023). Regardless, the solution to controlling ammonia is not as simple as increasing the cage-changing frequency, because cage changing is disruptive to the animals. A common approach to this challenge has been to develop new products that are better at controlling ammonia build-up, such as individually ventilated cages (IVCs) and novel bedding types. These allow for reduced cage-changing frequency while maintaining acceptably low ammonia levels and minimizing disruptions to animals, with the added benefit of reducing staff workload. Unfortunately, this approach ignores another often-overlooked stressor: the animals' motivation to avoid contact with their feces. For example, when male and female mice of several strains were tested in a four-cage setup, where one of the cages was clean and the others contained bedding that had been lived in for 1, 7, or 14 days, mice built nests and spent most of their time in the clean cage (Godbey et al., 2011). Additionally, as described in the **Housing** chapter, rats and mice housed in setups consisting of two or three clean cages moved nesting materials and bedding around to create one clean cage to nest in and another to use as a latrine (Amendola et al., 2023; Makowska et al., 2019). In contrast, in a conventional one-cage housing system, one study showed that there were no unsoiled resting areas within 4 days for

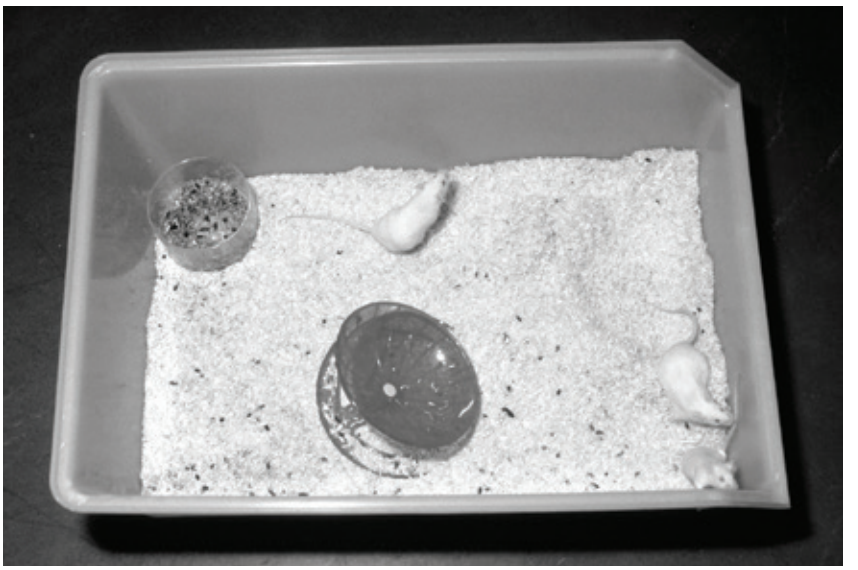

Figure 1: Mice provided with a demarcated area within their cage—such as a glass dish or a bottle—are likely to use the dish or bottle either for nesting or elimination, thus allowing them to better segregate their space into clean and dirty areas.

pairs and 3 days for trios of mice and within 3 days for pairs and 2 days for trios of rats (Boivin, 2013). If the cage-change interval is extended to 21 days, as some suggest (e.g., Felgenhauer et al., 2025), feces effectively *become* the bedding.

A potential solution for balancing ammonia, disruption to animals, and contact with feces would be to provide a physical separation of space within the cage. The area used for elimination could be changed frequently with minimum disruption to animals. For example, cages could be furnished with a litter tray or some kind of container, like a glass dish or bottle (Figure 1). These objects would almost certainly be used by rodents as either a latrine or nesting area, thus ensuring that the clean and dirty sites do not inadvertently mix as bedding gets displaced when animals move about the cage. As another option, the use of multi-cage setups would provide wholly separate living quarters while simultaneously increasing space and complexity. Such systems are available commercially or can be made in-house by connecting existing cages with short tunnels. For males or more aggressive strains of mice, cages could be connected using two or more tunnels to provide several escape routes and minimize the opportunity for a dominant individual to monopolize the tunnel.

Until the above solutions can be implemented, the appropriate cage-changing procedure should be determined on a case-by-case basis. Indeed, the rate of ammonia build-up in a given cage depends on the interplay of many factors, including the number, sex, and strain of the animals, the type and size of the cage, and the type and amount of bedding. Rather than review the impact of each factor separately (e.g., comparing the absorbency of two types of bedding), the cage-changing sections of this chapter will focus on the creative and sometimes unconventional ways to balance disruptions to the animals with varying cage, bedding, and animal-based parameters at a given facility.

When evaluating the appropriateness of any modified cage-changing regimens, it is important to measure ammonia levels objectively (i.e., with a sensor), because people tend to be poor at evaluating air quality within IVCs, and ammonia-induced pathologies are often clinically silent (Eskandarani et al., 2023; Vogelweid et al., 2011). Moreover, ammonia levels are not uniform across the cage; levels can be as much as three times higher by the latrine compared to the opposite side of the cage (Freymann, Tsai, Stelzer, & Hackbarth, 2017; Ulfhake et al., 2022).

Most studies investigating disruptions induced by cage changing have noted increases in physiological parameters that could, conceivably, have been caused by increased activity as animals explored their new environment. For example, in

mice, cage changing during the light phase caused a significant increase in daytime activity lasting several hours or days (Pernold et al., 2019; Ulfhake et al., 2022); an increase in heart rate, blood pressure (Gerdin et al., 2012), serum corticosterone (Rasmussen et al., 2011), and body temperature; and a reduction in time sleeping (Febinger et al., 2014). Similarly, in rats, cage changing led to increased activity, serum corticosterone, prolactin, blood pressure, and heart rate (Castelhano-Carlos & Baumans, 2009; Sharp et al., 2002, 2003). These changes aren't inherently bad or indicative of negative stress; in a world where the animals have little stimulation, an increase in activity (which can naturally lead to an increase in body temperature, cardiovascular parameters, and corticosterone) as animals explore their new environment and rebuild their space could actually be a positive thing.

However, there is some evidence to suggest that these changes are likely related to the stressful experience of handling during cage changing rather than the change in environment *per se*. Rasmussen et al. (2011) conducted two experiments to disentangle the effects of various factors involved in cage changing. In experiment 1, they found that serum corticosterone increased equally in all group-housed male C57BL/6J mice after cage changing—whether this involved transfer into a new clean cage or transfer back into the animals' old cage; for handling, mice were grasped with forceps anywhere along the body. In experiment 2, they found that after a change into a new clean cage, serum corticosterone increased in mice who had been actively handled (with forceps or by the tail with a gloved hand) but not in those who had been passively handled (allowed to hop into the new cage on their own after being gently herded with forceps) or not handled at all. These results suggest that it is handling—rather than placement in a new cage—that causes stress. A follow-up study should look at the effects of post-cage-change disruptions in mice handled using non-aversive methods (see chapter on **Human-Animal Interaction**). In rats, Sharp et al. (2003) found that heart rate increased significantly and equivalently in Sprague Dawley females, whether they were transferred into a new clean cage or placed back into their old cage. Thus, cage changing—perhaps largely related to handling—is stressful to mice and rats; this negatively impacts not only their welfare but also the validity of any data obtained from them in the hours or days after a cage change.

An added stressor that necessitates more frequent cage changing is flooding caused by leaky water bottles. One simple approach to minimizing the occurrence of cage flooding is to preassemble the clean cages—bottle included—a day or so before cage changing. Indeed, Rammling et al. (2014) observed that the majority of cage flooding incidents occur shortly after cage changing. To prevent exposing animals to leaks and floods from water bottles, they implemented a prescreening process where the

entire clean cage was assembled with the water in advance to help identify leaking bottles and pouches before introducing animals to the cage. As an added benefit, this preassembly method also reduced labor during cage changing.

Mice

CAGE CHANGING AND ROUTINE HUSBANDRY

As described in the Housing chapter, individually ventilated cages (IVCs) have become increasingly popular over the last two decades. Their use has increased not only because they offer several benefits to biosecurity, but also because they allow for a longer interval between cage changes by reducing intracage humidity and ammonia levels.

For example, using CD1 breeding mice in microisolator cages (7.75 x 12 x 6.5 in.) with 1.25 cups of corncob bedding, Carpenter et al. (2020) determined that acceptable ammonia levels (25 ppm) were exceeded after only 1 day for static-housed trios compared to 3 days for ventilated trios (and 2 days for static-housed pairs). Static cages holding pairs or trios were more humid, and 25% of their bedding was wet within 6 to 7 days; ventilated cages holding trios took a few days longer, while those housing pairs reached 25% wetness after 11 to 14 days. Pups weaned from static breeding cages had significantly more nasal lesions as a result of higher ammonia levels, with greatest severity in static-housed trios. Given that cage changing is stressful for mice, breeding pairs may be better than trios for maintaining a clean environment while minimizing disruptions to the breeding process and pregnant females, and ventilated cages may be beneficial if sufficient nesting material is provided (see chapter on Housing).

Jones et al. (2022) compared the effects of two IVC cage-changing regimens (14-day interval with spot changes as needed, or spot changing only) on intracage parameters and staff workload. Each cage (11.75 x 7.25 x 5 in.) housed four male CD1 mice on a mixture of woodchip and cellulose bedding and an igloo shelter. In both regimens, spot changing was performed only if the cage met established visual cleanliness criteria and consisted of changing only the cage and shelter, with the lid and filter top changed only if they were visibly dirty or damaged. The authors found that intracage temperature and ammonia levels did not differ between the two regimens, with 10 ppm as the highest ammonia level detected. The 14-day change regimen resulted in 302 cage changes (132 scheduled and 170 spot changes), compared to 270 changes in the spot-change-only regimen. Despite necessitating fewer cage changes, the spot-

change-only regimen was challenging for staff. Specifically, staff reported that the lack of scheduled changes meant they could no longer predict how much time they would need to spend on cage changing on any given day, which also made it more challenging to accurately allot staff time to other tasks. Staff also had concerns about "borderline cages" that didn't quite meet spot-changing criteria; when borderline cages were found, staff tended to change them even though they didn't meet criteria because they worried, among other things, that another staff member working in that room the next day may believe that the cage had already met criteria the day before but hadn't been changed, or that they would be leaving extra work for another staff member the next day. Accordingly, a third cage-changing regimen was implemented: A hybrid "scheduled spot change" method consisting of daily spot changes with a scheduled evaluation every 14 days. Cages that were borderline during the scheduled evaluation were also changed at this time. Staff agreed that the addition of focused evaluations helped address the management of borderline cages and facilitated the return of more predictable cage-changing patterns.

Partial cage changing can be implemented to retain existing scent marks of mice, drastically reduce the amount of cage washing conducted, and potentially reduce animal stress related to cage changing. Taylor et al. (2019) compared partial bedding replacement (75% of the dirty bedding was replaced every 2 weeks, with the cage bottoms replaced every 16 weeks) with full cage changes every 2 weeks in group-housed male C57BL/6 mice. When corncob bedding was used, the partially replaced cages had higher ammonia levels than the fully changed cages, but there were no differences when paper-based bedding was used; notably, all cages remained below 25 ppm regardless of bedding or cage-changing method. There were no differences in animal behavior, animal appearance, humidity, or levels of ATP (a nucleotide found in the cells of all plants and animals, including bacteria, and used here to evaluate sanitation) based on cage-changing method. Anecdotally, the researchers noted that mice in the partial replacement group seemed calmer after changing.

Retaining used nesting material during cage changing is another method that helps maintain odor cues within a cage and reduce aggression between mice (see chapter on **Social Housing**). Barabas et al. (2019) confirmed that after 1 week in a mouse cage, nesting material contained a variety of proteins from saliva, urine, and sweat; because these proteins are used by mice for scent marking, retaining some of the old nesting material helps to maintain important odor cues within the cage.

Modern technology can also be used to determine when cages need changing. Filby (2020) described how their facility collaborated with the manufacturer of their

digital ventilated rack system to develop an algorithm that automatically flags when a cage base needs cleaning. The digital rack system is primarily designed to allow for automatic tracking of mouse movements within the cage, but it can be adapted to flag cages that need cleaning. The algorithm considers moisture level, time since last bedding change, and number of animals in the cage. They aimed to strike a balance between air quality, proportion of wet bedding, and availability of feces-free areas within the cage.

Freymann et al. (2015) tested the preferences of female group-housed C57BL/6 and BALB/c mice for different depths of aspen chip bedding. Using a two-cage system, the authors compared the amount of time mice spent in each cage within the following bedding depth pairings: 0.2 versus 0.6 inches, 0.2 versus 2.4 inches, and 0.6 versus 2.4 inches in Makrolon Type III cages (14.8 x 8.5 x 5.9 in.). In each instance, both strains spent significantly more time in the cage that contained more bedding, and the preference was strongest when one of the cages was bedded to a depth of only 0.2 inches. The authors noted that the ability to perform daily health checks was not impacted even with the deepest bedding, because mice created small hollows rather than tunnels they could hide within. Freymann, Tsai, Stelzer, and Hackbarth (2017) replicated these results in males and females of the same two strains, except that, in their study, the C57BL/6 males' preference for a bedding depth of 2.4 inches versus 0.6 inches did not reach statistical significance. Furthermore, they found that when mice were group housed within a cage bedded to 0.2, 0.6, or 2.4 inches, males and females with the lowest bedding depth had lower body temperatures, consumed more food, engaged in more nest-building behavior (this was defined as arranging, pulling in, and fraying of *bedding* material, since mice were not given nesting material), and their cages reached higher ammonia levels, which were measured on day 7. Finally, using the same animal and housing conditions, Freymann, Tsai, Stelzer, Mischke, et al. (2017) found that, under deeper bedding conditions, each of the four groups (2 sexes x 2 strains) displayed some combination of the following indicators of warm adaptation: smaller adrenal, kidney, liver, and heart weights and larger body and tail lengths. Ammonia levels were highest in the shallowest bedding condition. Taken together, these results suggest that in the absence of nesting material, deeper bedding allows mice to better thermoregulate while also contributing to lower intracage ammonia levels.

Contrary to the studies led by Freymann, Eskandarani et al. (2023) found that increasing the amount of aspen chip bedding within Makrolon Type II or III cages did not reduce ammonia levels, which were measured on days 7 and 14, though ammonia levels were lower in the larger (Type III) cages. In this study, the cages were furnished

with shelters, cardboard tubes, nesting materials, and gnawing sticks, while cages in the studies led by Freymann were barren. Eskandarani et al. noted that empty cages are used to test and calibrate airflow in IVCs. However, when these cages—with their low headspace—are furnished with shelters and other materials, airflow may be diverted, resulting in ineffective ventilation, higher moisture levels, and subsequent ammonia build-up. They concluded that factors like cage provisioning and cage geometry likely have a substantial effect on ammonia levels.

Others have also documented the benefits of using more bedding. In a descriptive study, White (2012) noted that mice of 22 strains derived several benefits from being housed on 1 versus 0.13 inches (the latter was the industry standard in 2011) of aspen chip bedding. Namely, mice spent more time foraging and burrowing, fought less, and were less disturbed thanks to less frequent cage cleaning. Moreover, breeding females were observed building ramps under the food hopper that allowed mobile pups to access the food hopper while simultaneously allowing the dam to separate herself from demanding pups. Later, White (2019) documented ammonia levels in cages bedded to a depth of 0.6 versus 1 inch of Lignocel Select bedding. A cage containing five females would reach ammonia levels of 25 ppm within 5 days when bedded to a depth of 0.6 inches compared to 12 days when bedded to a depth of 1 inch. A cage containing four males would reach these ammonia levels within 6 and 10 days when bedded to a depth of 0.6 and 1 inch, respectively. Moreover, deeper bedding decreased the rate of cage cleaning performed by technicians by 66%, resulting in less stress related to cage changing for the mice and financial savings due to reduced labor costs for the facility. Finally, increasing bedding depth to 1 inch did not cause more flooding; and when flooding did occur, mice were better protected from the spill because the bedding absorbed more water.

Even if animals are not handled, other room activities, such as health checks and cleaning activities, may impact the behavior and welfare of animals in the room, especially if their sleep is disrupted. Robinson-Junker et al. (2018) tested whether 1 hour of mild disturbances (visual welfare checks, supplementing food and water in cages, cleaning in the animal housing room) were more disruptive to mice if they occurred during the light or the dark phase. Using single-housed C57BL/6, BALB/c, and CD1 mice of both sexes, they found that the timing of mild husbandry disturbances had no effect on the overall amount of sleep, but it did affect sleep fragmentation (sleep bout length) differentially by strain and sex. In a follow-up experiment, Robinson-Junker et al. (2019) examined the effects of predictable versus random husbandry-related disturbances on mice. Daily for 6 days, single-housed C57BL/6NCrl mice of both sexes were subjected to four of eight possible

disturbances within the animal housing room, each lasting 15–60 minutes: the presence of a stranger, a conversation, pop music, cage-changing noises, a running cage-changing station with ventilation hood, running water, floor disinfection, and the presence of a t-shirt that had been worn by a human male. In the predictable treatment, disruptions occurred at the same time each day within two 1-hour windows (one in each phase of the light:dark cycle). In the unpredictable treatment, disruptions occurred at intervals of 45, 60, 90, or 120 minutes throughout the light:dark cycle. There was no effect of treatment on sleep disruption (percentage of time sleeping) or sleep fragmentation (sleep bout length). There also was no effect on chronic stress (measured as adrenal cortex:medulla ratio), but the authors conceded that the disruption period may have been too short to induce chronic stress. While these mild room-level disturbances did not have very significant effects on mouse sleep and welfare, it is likely that cage-level disturbances (e.g., pulling the cage for health checks, cage changing, mouse handling) would have more of an impact.

The choice of cleaning products may also impact mice, given that they are particularly sensitive to olfactory stimuli. Lopez-Salesansky et al. (2021) found that cup handling male and female C57BL/6 and BALB/c mice while wearing nitrile gloves that had been sprayed with an alcohol-based hand sanitizer (67% ethanol, 3% methanol, 30% water) resulted in various behavioral changes compared to handling with unsprayed gloves. Specifically, in a hand interaction test immediately after handling, neither treatment caused avoidance of the hand, but BALB/c mice who had been handled with the sanitized gloves engaged in significantly more sniffing and grooming of their cagemate, and both strains engaged in more self-grooming and wall rearing. After returning to the home cage, mice who had been handled with the sanitized gloves engaged in more self-grooming in the caudal region. Moreover, males handled with sanitized gloves displayed less aggression for 20 minutes after returning to their home cage, but it is unclear if aggression was truly reduced or simply delayed because mice were occupied with self-grooming. These findings indicate that alcohol-based sanitizer affects mouse behavior, including social behavior, but specific welfare impacts are unclear. In another experiment, Grau et al. (2019) found that male and female C57BL/6JRj mice placed in a testing arena avoided the location that contained a gauze soaked in 99% ethanol (they did not avoid the location when it contained an unsoaked gauze). When the ethanol-soaked gauze was present, mice also produced more fecal boli and reduced their locomotor activity. Additionally, Hershey et al. (2018) found that when given the choice to avoid common cleaning agents—isopropyl alcohol, chlorine dioxide, and bleach—male C57BL/6J mice avoided all three agents in a two-choice light–dark box test, with isopropyl alcohol being most aversive. (The two-choice light–dark box consists of a central light chamber with a dark chamber

on each side; here, one of the dark chambers contained a small receptacle with water and the other chamber contained a small receptacle with one of the cleaning agents.) The cleaning agents did not impact mouse behavior in the elevated plus maze test, indicating that these agents can be used to clean the apparatus between animals and reduce the potential spread of disease; however, given that these agents are aversive to mice, this finding may simply indicate that the novel elevated plus maze test environment is more stressful to mice than the cleaning agents.

INDIVIDUAL IDENTIFICATION METHODS AND GENOTYPING

A number of methods used to identify mice can simultaneously be used to genotype them. These methods are often invasive, such as ear punching or notching and toe clipping. Wever et al. (2017) performed a systematic review of studies that assessed the discomfort caused by toe clipping and ear punching, notching, or tagging in mice and rats. For toe clipping, four of the five studies identified were on mice. The main impacts of toe clipping were found to be decreased motor activity, reduced grip strength, and reduced swimming ability. Toe clipping is highly invasive and is strongly discouraged. For ear punching, notching, or tagging, six of the seven studies identified were on mice. The main impacts of ear punching, notching, or tagging were increased respiration rate, vocalization, and blood pressure (Figure 2).

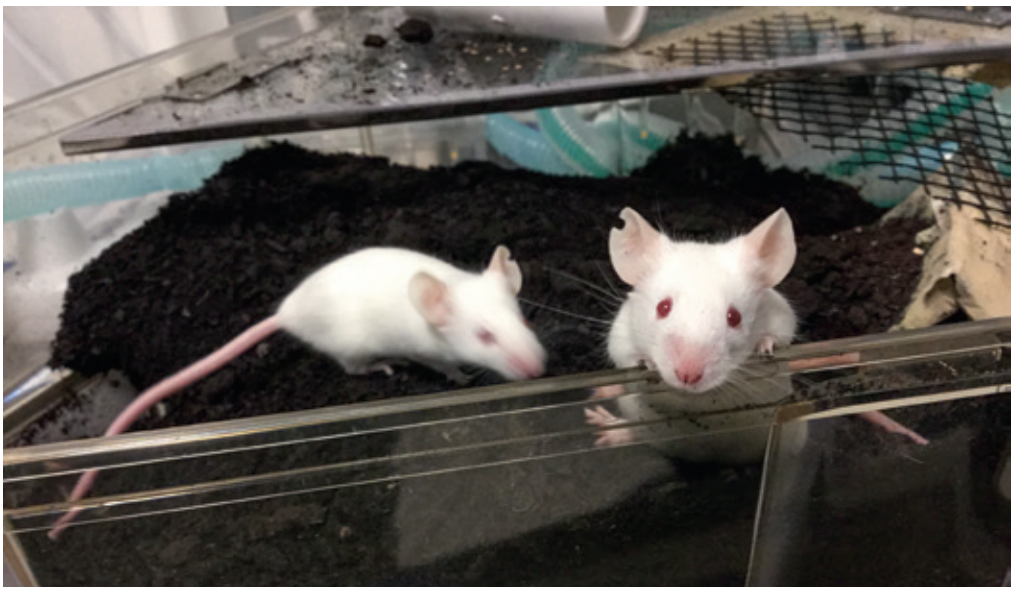

Figure 2: Ear punching, notching, and tagging are painful to mice, causing both short-term and long-term pain or discomfort—even when a local anesthetic cream is used during the procedure.

Although ear punching is less invasive than toe clipping, it is still painful. Taitt and Kendall (2019) found that female Swiss Webster mice subjected to ear punching groomed their head significantly more than those who were not ear punched; this behavior, which can indicate a response to pain, began to decline after 60 minutes. Ear-punched mice also had an elevated heart rate for 30 minutes and higher body temperature for at least 60 minutes after the procedure compared to baseline. These measures did not differ from baseline in mice who had experienced only restraint (via scruffing) or other routine husbandry procedures. Burn et al. (2021) compared the effects of ear punching to weekly tail marking with a permanent marker in weanling male BALB/c mice. They found that ear punching, both with or without a local anesthetic cream, caused signs of pain and anxiety. Over the course of 5 weeks, mice who were ear punched were less likely to approach a handler, less likely to eat novel food, and spent more time grooming their bodies and ears than tail-marked and unmarked mice. Mice marked weekly with a permanent marker showed no differences in these behaviors from unmarked mice. All marking procedures were acutely stressful, since mice defecated during their occurrence, but defecation decreased over time when tail marking was reapplied. Ear punching may also cause pain or discomfort as the tissue starts to heal. Indeed, Gaskill et al. (2017) found that 2 weeks after being subjected to a similar procedure—ear notching using scissors—male C57BL/6 mice had higher pelt aggression lesion scores (indicative of more injurious aggression, which is known to be exacerbated by stress) than those who had been subjected to tail tattooing.

Ear tagging (i.e., piercing of the ear with a metal tag) and tattooing are also painful in the short term. Roughan and Sevenoaks (2019) found that 20–25 minutes after being subjected to ear tagging, male and female BALB/c mice grimaced more than mice who were tattooed on the tail; however, tail tattooing did cause acute agitation and tail-base inflammation. The ear tags and tattoos were later used in an identification exercise; all mice who were ear tagged required handling and restraint for the tag to be read correctly, and some were misidentified, while all tattoos were read correctly without handling the mice. Tail tattooing, however, can cause other post-procedural complications. Young et al. (2020) were concerned about the occurrence of ulcerative dermatitis in some mice after tail tattooing. After investigating bacterial and heavy metal content of tattoo ink and tattooing equipment, they found that both opened and unopened containers of tattooing inks were contaminated with environmental microbes (e.g., *Enterococcus*). Furthermore, the red ink contained very high levels of lead and arsenic, which caused immediate skin irritation in the mice tattooed with this color. Taken together, these studies suggest that the least invasive method to mark mice is weekly tail marking with a permanent marker, although this does

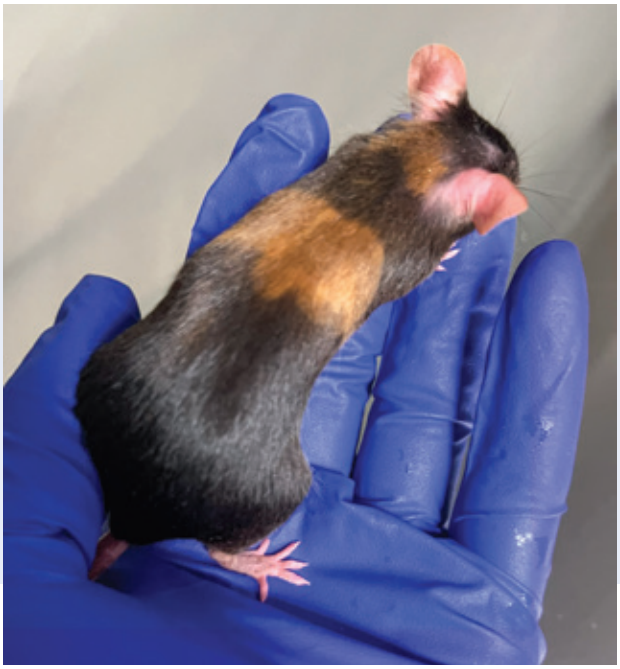

Figure 3: Fur bleaching and dyeing is a relatively noninvasive method to mark mice, lasting several weeks.

require ongoing maintenance. All manners of ear identification (punching, notching, and tagging) and tail tattooing cause acute and longer-term pain; tail tattooing, meanwhile, appears to cause less acute and long-term pain than ear identification methods but may result in post-procedural complications.

Another relatively noninvasive method that requires less frequent reapplication than tail marking is fur bleaching and dyeing (Figure 3). Gouveia and Hurst (2013) used home hair dye kits to mark mice on the shoulder or rump regions, using a Clairol Nice and Easy black dye on white ICR mice and a Jerome Russell blonde dye on black C57BL/6 mice. Mice were restrained on a wire cage lid, and the dye was applied using a small paint brush. Mice were then placed back in their cage; after 20 minutes, the dye was washed off using cotton wool soaked in warm water. Mice were dried off with another piece of cotton wool. Fur dye stays on for several weeks.

More humane methods to genotype mice also exist. Otaño-Rivera et al. (2017) demonstrated that mice can be genotyped using genomic DNA purified from hair roots. Mice were placed in a clean cage, and the hair sampling site was

decontaminated with alcohol. Sterilized forceps were used to pluck 10–20 hairs from each mouse. The authors found that Chelex 100 Resin (a cation chelating styrene divinylbenzene copolymer) worked better—with fewer failed reactions—than Proteinase K (a serene protease enzyme) to extract DNA from the hair roots. Quality of DNA extraction and resulting genotypes from hair samples were comparable to outcomes from tail-tip tissue samples.

Skin swabs represent an even less invasive genotyping method. In one study, Okada et al. (2017) swabbed the ventral or dorsal skin of adult mice, wiping three times in the opposite direction of hair growth. These samples were used for DNA isolation and polymerase chain reaction (PCR) analysis. Findings from skin swabs were the same as those obtained through tail-tip amputation. Finally, Huang and Kayne (2020) described their successful use of buccal swabbing to genotype mice. The inside cheeks of mice were swabbed 15–20 times using an autoclaved microbrush, which provided a small quantity of DNA that was amplified using the quantitative PCR (qPCR) process. The authors found that the key to optimizing detection of genes via small buccal samples was to work with short DNA fragments (below 200 base pairs). The authors also compared the buccal-swab method to conventional tissue sampling for several hundred animals, and found 100% concordance, even though tissue samples provide comparably greater DNA concentrations.

Rats

CAGE CHANGING AND ROUTINE HUSBANDRY

The benefits of transferring olfactory cues to reduce stress during cage changing are more widely studied in mice because mice are more likely to show aggression after the change. However, the comparatively lower tendency of rats to fight after cage changing does not necessarily mean they wouldn't benefit in other ways from the retention of olfactory cues. To test whether the transfer of olfactory cues affects rats' stress during cage changing, Meller et al. (2011) measured cardiovascular parameters via implanted telemetry in group-housed male Sprague Dawley and Wistar rats following four procedures: Rats were gently lifted by the base of the tail and either (1) placed back in the old cage, (2) transferred into a new cage with new tunnel and new lid, (3) transferred into a new cage with old tunnel and new lid, or (4) transferred into a new cage with new tunnel and old lid. Rats experienced elevated heart rate and mean arterial blood pressure for at least 5 hours after each of the procedures, with the biggest elevations in the first hour after the procedures. However, these parameters were least elevated in the rats who were returned to their old cage and most elevated

for those who were placed in a new cage with new tunnel and new lid. In Wistar rats, transferring the old lid resulted in lower cardiovascular changes than transferring the old tunnel; in Sprague Dawley rats, transferring either the old lid or the old tunnel resulted in similar cardiovascular changes. Taken together, these results indicate that, overall, transferring the old lid is a comparatively more effective way to reduce disturbance-related stress caused by cage changing in these albino rat strains.

As another alternative for transferring odor cues during cage cleaning, Henderson (2020) suggested sifting corncob bedding using a sieve to remove rat feces, allowing for reuse of dry bedding and cages for up to 3 weeks. Prior to sifting, nesting material and wet patches should be removed. This method reintroduces animals to an environment with a familiar scent while also reducing waste (Figure 4). It does, however, present some risk to workers through exposure to dust from dirty bedding and, over time, may impact ammonia levels.

Figure 4: Henderson (2020) described using a sieve to remove rat feces from bedding; this method keeps rats' familiar scent, while allowing the re-use of dry bedding for up to 3 weeks and reducing waste. Left: Before sifting, after removal of all dirty nesting material; Right: After sifting.

Abou-Ismail et al. (2015) assessed how group-housed male Wistar rats were affected by witnessing other rats in the room undergo routine husbandry procedures (cage changing, weighing, tail marking, restraint, and injection) during the light versus dark phase of the light:dark cycle. The rats witnessed other rats undergo the procedures 2–3 times per week for 5 weeks but did not undergo the procedures themselves. Rats witnessing husbandry procedures 3–4 hours after the lights came on had significantly more indicators of low mood and chronic stress than those who witnessed the procedures 3–4 hours after the lights went off. Specifically, they had lower body and thymus gland weight; had higher adrenal gland weight; spent less time sleeping and interacting with enrichment; and spent more time feeding, interacting with bedding, and engaging in agonistic and inactive-but-awake behaviors. The authors suggested that the occurrence of these procedures during the light phase is likely more stressful due to the disruption of circadian rhythms and the possibility that these nocturnal, albino animals are more sensitive to stress in the light. As a refinement, they recommend that experimental and husbandry procedures should be conducted during the dark phase and outside of the housing room.

INDIVIDUAL IDENTIFICATION METHODS AND GENOTYPING

Kasanen et al. (2011) compared the effects of ear tattooing, ear notching, and microtattooing on cardiovascular parameters (which can indicate disturbance, stress, or pain) in group-housed male Sprague Dawley and Wistar rats. Ear tattooing was performed using tattooing pliers; ear notching was done using Kopferdam pliers to make two holes on the periphery of the ear; and microtattooing was done using the Aramis microtattoo system to pierce the distal toe pad of both third phalanges on the rear feet. Each identification procedure lasted less than 1 minute and was performed standing next to the animals' home cages. During the first hour after the procedures, both heart rate and blood pressure were significantly higher in rats who had received an ear tattoo compared to those receiving either of the other two procedures. Thereafter, differences were more variable: From 1 to 4 hours after the procedures, blood pressure was *lower* in the ear tattoo group compared to the other two groups, while no heart-rate differences were observed between groups. During the dark period 4–16 hours after the procedures, blood pressure was substantially lower in the toe microtattoo group compared to the other two groups, with again no differences in heart rate between groups. Finally, in hours 16–24 after the procedures, blood pressure was again lower in the toe microtattoo group compared to the other two groups, while heart rate was highest in the ear tattoo group and lowest in the ear notch group. The authors concluded that (toe) microtattooing may be a refinement over ear tattooing or ear notching since, overall, it caused smaller changes in heart

rate and blood pressure. However, because the location of the microtattoo was different from the other two methods (toe vs. ear), it is unclear whether location or technique had a greater effect on the outcome.

All three methods described above cause some degree of pain. We recommend the use of noninvasive marking methods, such as using scissors to cut, or clippers to shave, patches of fur (Boulanger Bertolus et al., 2015) or using a permanent fur marker, such as the Animal Marker by Stoelting (Makowska & Weary, 2016) or the Livestock Marker by Ketchum (Améndola et al., 2019). While permanent markers used on the tail need weekly application, those formulated specifically for animal fur stay on for 6–12 weeks.

Other Rodents

CAGE CHANGING AND ROUTINE HUSBANDRY

McCullagh et al. (2017) tested whether a prolonged cage-change interval would be appropriate for pairs of **Mongolian gerbils** housed in IVCs (15 x 19.5 x 8.5 in.; 58 air changes per hour) bedded with aspen chips. They found that temperature and humidity were significantly higher at cage changing when the interval was 6 weeks compared to 2 weeks. However, ammonia levels at cage changing did not differ, and in the two animals sampled per treatment for this measure, no abnormalities were observed in nasal histopathology. There were also no differences in reproductive performance (number of litters, number of pups per litter) or pup weights at weaning. The authors concluded that longer cage-change intervals were beneficial since they reduced the frequency of stressful events, although they did not assess the potential stress the animals may have experienced from the increased amount of feces in their cage over time.

Veillette and Reebs (2010) assessed male and female **Syrian hamster** preferences for nesting in clean or dirty cages. First, hamsters were single housed for 10 days in a system of two open-top cages (16.5 x 8.7 x 8.3 in. each) connected by a tunnel. Then, both cages were rebedded with 4 cups of fresh pine shavings, and the tunnel was blocked such that they were restricted to one of the cages. After 3, 9, or 14 days, the tunnel was unblocked and the authors observed which cage the hamsters chose to nest in: the cage they had occupied during the interval or the other cage containing unsullied bedding. In both sexes, at least 75% of the hamsters nested more in the lived-in cage. The authors concluded that a clean cage with fresh bedding is only a little attractive to hamsters when their current bedding is up to 14 days old; possibly,

at some point beyond the maximum 14 days studied, the cage containing fresh bedding would become preferable.

ANIMAL GROOMING PROCEDURES

Guinea pigs require regular nail trimming as part of their grooming. McMahen and Williams (2018) suggested trimming nails frequently to keep foot pads healthy and to avoid the blood supply in the nail growing closer to the tip. If a guinea pig is reactive to handling, they recommend trimming only one or two feet per day and having one person hold the animal while another person trims. Once a handler is comfortable with holding and trimming at the same time, the nondominant hand can be used to hold the guinea pig with the animal's back against the handler's chest, which should result in the animal naturally spreading their toes apart. Once finished, treats should be offered so that the animal builds a positive association with nail trimming.

LaFleur and Williams-Fritze (2020) explained that **chinchillas** require several dust baths per week to prevent their fur from becoming matted and greasy (Figure 5). Dust baths should be removed once the animals have cleaned themselves, because too

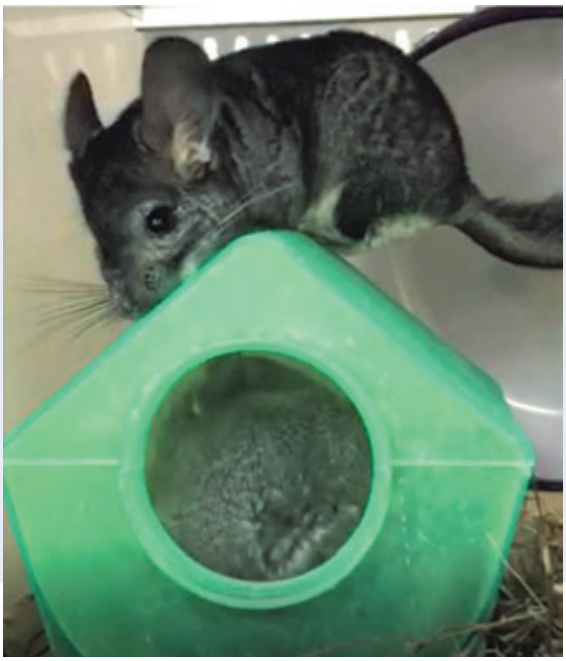

Figure 5: Chinchillas must be provided with a dust bath regularly to maintain clean fur. Baths should be removed after use to reduce the chance of the animals developing conjunctivitis.

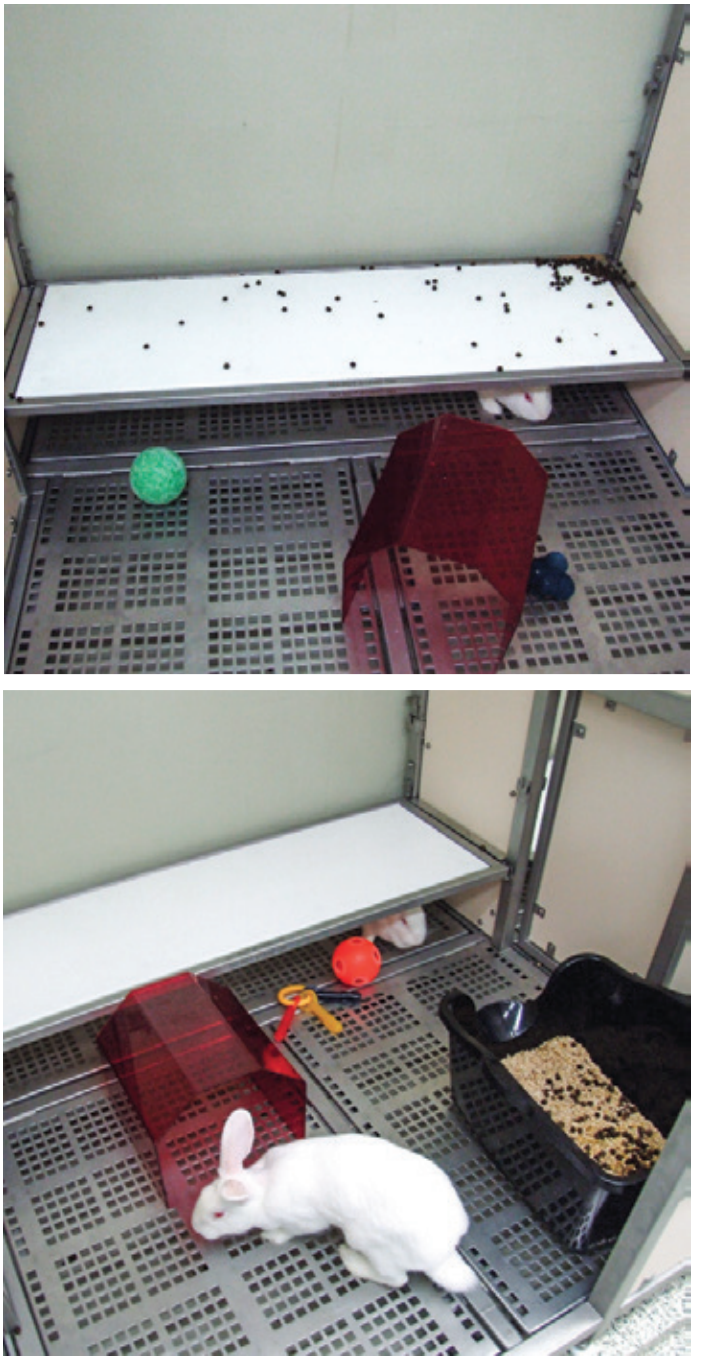

Figure 6: Merrill and Ordile (2016) described training rabbits to use a litter box, which helps to keep their pens clean and saves time on husbandry. Top: Standard rabbit pen after 8 hours; Bottom: Rabbit pen after 8 hours when a litter box was provided.

much bathing can lead to conjunctivitis. Participants in AWI's online discussion forum (LAREF) uniformly agreed that providing chinchillas with access to a dust bath is a must (Reinhardt, 2020). One participant shared that they provide dust baths daily in a small cat litter box, while another shared that they provide them for 30–45 minutes at least twice a week in a mouse shoebox cage.

Rabbits

CAGE CHANGING AND CLEANING METHODS

Providing pen-housed rabbits with a litter box helps keep their living areas noticeably cleaner (Figure 6). Merrill and Ordile (2016) described how New Zealand white rabbits are trained to use a litter box at their facility. When rabbits first arrive, two boxes are placed in their pen; if needed, the boxes are relocated to where the rabbits have chosen to eliminate. After about 5 days, the box that is least used is removed permanently. The authors recommended using Nalgene boxes (15 x 8.53 x 5.5 in.) rather than conventional cat litter boxes, as they can withstand chewing and sanitization in a cage washer. Litter boxes are filled with corncob bedding, since this substrate is believed to be safe for rabbits to ingest and does not clog facility drains. Boxes are cleaned twice a week and filled with fresh bedding and a handful of dirty litter to keep the animals accustomed to eliminating within the box. The implementation of litter boxes was shown to save 13 minutes on daily husbandry and 1 hour on bimonthly "breakdown" (floor to ceiling sanitization) in each room, in addition to the benefit of keeping animals out of contact with their urine and feces.

INDIVIDUAL IDENTIFICATION METHODS

Tattooing causes acute pain in rabbits and should not be performed without analgesia. Keating et al. (2012) demonstrated that male and female New Zealand white rabbits undergoing ear tattooing without the use of a local anesthetic cream exhibited significantly more struggling and vocalizing, increased heart rate and blood pressure, and higher grimace scores compared to rabbits pretreated with the cream (a mixture of prilocaine and lidocaine). A thin layer of the cream was applied 20 minutes before the procedure. Therefore, topical analgesia should be used prior to tattooing and may be safer and more accessible than general anesthesia for this procedure.

KEY TAKEAWAYS: COLONY MANAGEMENT

- Colony management requires striking a balance between maintaining animal health and hygiene and minimizing the disruption and distress induced by the associated procedures.
- The most effective way to strike this balance for **rodents** and **rabbits** is to provide the animals with segregated spaces, such as interconnected cages or litter trays, so that animals can defecate in an area separate from where they sleep. Mice, rats, and rabbits naturally segregate their space into clean and dirty areas, and the animals themselves prefer this arrangement. This also allows animal care staff to change the sullied area as animals rest undisturbed in a different area.
- When single-compartment cages are used for **rodents**, familiar odor cues can be maintained during cage changes by retaining nesting material, shelters, and/or cage lids.
- The frequency of cage changes can be reduced by providing animals with more bedding material. **Rodents** prefer deeper bedding, which can aid in nesting and thermoregulation and provide better absorbency in the event of cage flooding. More bedding may also be associated with lower ammonia levels.
- IVCs allow for prolonged intervals between cage changes, which can reduce stress related to cage changing. However, a prolonged cage-change interval may negatively impact welfare because it leads to the accumulation of fecal matter in the cage over time, resulting in the absence of clean resting areas within the cage.
- Creative or unconventional cage-changing regimens—such as spot-cleaning, partial cage changing, or sifting dirty bedding—reduce disturbances to **rodents** while maintaining adequate cage cleanliness.
- **Mice** have demonstrated aversion to alcohol-based products, with notable changes in their behavior after exposure to alcohol. Isopropyl alcohol may be more aversive than bleach or chlorine dioxide cleaning products. Rodent exposure to cleaning agent scents should be minimized.
- Ear punching and tail/ear tattooing cause short- and long-term pain and other complications in **rodents**. More humane alternatives include hair dyeing, hair clipping, or hair marking with a permanent animal fur marker (lasting several weeks) or tail marking with a permanent marker (lasting 1 week). If ear tattooing is conducted on **rabbits**, a topical analgesic cream should be used.

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Transportation

What Is It, And Why Does It Matter?

A large number of rodents and rabbits are transported by road or plane, for hours or days, before arriving at a research facility. While the journey itself is very stressful, myriad other transport-related factors compound the animals' stress. A report by the Laboratory Animal Science Association (LASA) Transport Working Group (Swallow et al., 2005) identified 11 potential sources of stress for animals before, during, and after transport:

- Handling of animals
- Separation from familiar cagemates; possible single housing during journey
- Confinement in an unfamiliar container
- Procedures associated with loading and unloading the container
- Movement and vibrations during the journey (including acceleration and deceleration)
- Physical stress due to maintaining balance
- Unfamiliar sights, sounds, and smells
- Fluctuations in temperature and humidity
- Withholding of food, or voluntary abstention from food or water
- Disruption of the light:dark cycle
- New housing and care protocols at destination, including new (unfamiliar) humans and new (unfamiliar) social groupings or hierarchies

An additional risk to welfare is that it can be difficult, if not impossible, to monitor the animals for disease or injury during the journey. This is all the more problematic because stress can weaken the immune system and exacerbate the risk of disease.

After arrival at the research facility, animals must be given an adequate acclimation and stabilization period to allow them to recover from the stressful journey and adjust to their new environment. The same care should be taken after transferring animals between rooms within a facility. This is not only to mitigate the effects of cumulative stress but also to ensure that the animals are physiologically and behaviorally normal before they are used in research. Indeed, transport stress affects animals' ability to cope with the transition to a new laboratory environment, and may therefore impact their subsequent enrollment in studies and the validity of experiments conducted on them.

Surprisingly few studies have been published on transport-related stress in rodents and rabbits, with little data on what constitutes an appropriate acclimation period for

each species. What little research exists is mostly decades old and largely focused on single acute physiological changes in the first few days after transport (see Conour et al. (2006) for a review). Whole-animal impacts of transport—particularly on behavior or other indicators of health and welfare—are not well studied in animals shipped to laboratories; however, a few recent studies have begun to address these issues.

Summaries of Current Refinement Research

Mice

Rumpel et al. (2019) measured fecal corticosterone metabolites in 3-week-old female mice of five different strains (C57BL6/J, C57BL/6NCrl, FVB/NCrl, Crl:CD1(ICR), and BALB/cAnCrl) after a journey of over 375 miles by road that lasted more than 22 hours from packing to unpacking. The prepubescent mice were transported in groups inside unlit, air-conditioned trucks, within filtered transport crates with bedding, nesting material, food, and a source of hydration (HydroGel). A few hours after arrival, fecal corticosterone metabolites were elevated in all strains except FVB/NCrl, although the elevation was statistically significant only in C57BL/6NCrl mice. Values were back to pre-packing and pre-transport levels on day 4.

Colloidal water gel cups commonly used as a source of hydration during transport are typically placed on the bedded floor of the transport crate. Locklear and Whiteside (2021) noticed that the gel cups often get buried in bedding and feces, especially with days-long international shipments that may be further prolonged if there are delays with the airline or customs. Sometimes the gel has evaporated by the time of arrival. To address these problems, the authors experimented with different types of gel containers and their placement within the crate. Based on feedback from their domestic and international collaborators, they found that water gel pouches hung on the wall of the crate, with an X-shaped slit cut into them to provide access to the gel while preventing it from falling out, worked best (Figure 1). The hanging gel pouches stay in place with minimum evaporation and contamination from bedding and feces.

Figure 1: Locklear and Whiteside (2021) found that hanging water-gel pouches on the transport box wall with an X-shaped slit cut into them minimized evaporation and contamination from bedding and feces.

Focusing on transportation within an animal facility, Cordingley et al. (2024) evaluated behavioral and physiological measures in single- and group-housed male C57BL/6J mice after they were transported for 15 minutes between two housing areas within the facility; the route was on even epoxy resin flooring and included a ride in an elevator. Prior to transporting the animals, the authors tested various transport devices (flatbed, stainless steel rack, plastic cart, plastic cart with pneumatic wheels, metal cart) to determine which were associated with the least and the most noise and vibration. They found that a plastic cart with pneumatic wheels produced the least average and maximal noise and vibration, while the metal cart produced the most; these two carts were then used to transport mice. There were no behavioral differences in the open field and elevated plus maze tests between mice transported via one method versus the other. However, compared to untransported controls, mice transported on the metal cart had elevated plasma corticosterone levels during the first 48 hours after transport (and possibly longer, as no samples were taken beyond 48 hours). The noise and vibration from metal carts should be regarded as a stressor that may impact mice for days; using carts with pneumatic wheels or carrying cages by hand can reduce exposure to stressful noise and vibrations and may improve the validity of research data.

Rats

Arts et al. (2012) performed a comprehensive examination of the physiological and behavioral consequences of road transportation in rats. Six-week-old male HsdCpb:WU

(Wistar) rats were placed with their cagemates into plastic transport boxes with bedding, food pellets, and water gel (HydroGel) and driven by van for approximately 3 hours from the breeding facility to the research institution. The temperature at their origin (68–72 °F; 20–22 °C) was warmer than it was at the packing and holding area (63 °F; 17 °C) and the truck (59 °F; 15 °C). A control group was packed into the boxes but not transported, and another control group was untouched. Rats who had been packed—with or without transportation—showed a decrease in body weight that returned to levels equal to that of the unpacked group after 1 week. Plasma corticosterone was elevated in both packed groups and, in the transported animals, was not yet back to baseline 16 days after transport, when the last blood sample was taken. Heart rate and mean arterial pressure both decreased during transport and had not returned to baseline 21 days later, when the last measurements were taken; instead, both stabilized at a new, lower value 4 days after transport. Self-grooming—a self-soothing behavior that generally increases in response to mild stressors—was higher in transported rats and took 1 week to return to baseline. Most notably, transported rats stopped engaging in play-related behaviors and did not resume until 2 weeks after transfer. In sum, this study demonstrated that packing and transport are both significant stressors for male rats; some parameters returned to baseline within 1 or 2 weeks, others stabilized at a new level within 1 week, and others neither stabilized nor returned to baseline in measurements taken up to 3 weeks after transport.

In a follow-up study, the same researchers investigated the effects of between- and within-facility transportation on 3-week-old male and female HsdCpb:WU (Wistar) rats (Arts et al., 2014). Transport conditions from the vendor to the research facility were identical to those described above, except this time the journey lasted approximately 6 hours due to multiple stops for delivery of other animals. For within-facility transport, rat cages were placed on a cart for approximately 30 minutes, driven for 5 minutes to another room, and placed on a new cage rack. Rats transported between facilities experienced weight loss, increased plasma corticosterone, and increased activity; heart rate increased in the females but decreased in the males. Based on when parameters returned to baseline or stabilized, the authors recommended that, after road transport from the vendor, males be given 8 days and females be given 2 weeks to acclimatize. For transfer between rooms within the same facility, both sexes should be given 2 days to acclimatize before starting experiments.

Simpson (2017) assessed how long male Han Wistar rats took to acclimatize to a new facility after 6 hours of transport in a van inside transport boxes supplied with food and water gels (HydroGel). Rats lost 8% of their body weight during transfer and took 3 days to regain the lost weight. Although rats are usually more active at night, these

rats were initially more active during the day, and dark period activity did not stabilize until night 5, highlighting the disruption to their circadian rhythm.

Other Rodents

Walters et al. (2012) investigated changes in fecal cortisol in male **Hartley guinea pigs** following a 4-day journey on a truck from a vendor to the animal research facility. The guinea pigs were transported in "group compartments." Once they arrived at the research facility, they were rehoused either singly or with a social partner, and either with or without a hut. In the single-housed animals, the hut had no effect on fecal cortisol levels; in the pair-housed animals, fecal cortisol was significantly lower in animals with a hut. In all groups, fecal cortisol was still increasing after 4 days of acclimatization to the new facility. The authors concluded that male guinea pigs should be pair housed with a hut and allowed more than 4 days to become comfortable and stable following the stress of transport to a new facility.

Rabbits

Peric et al. (2017) assessed stress levels in single-housed female New Zealand white rabbits after they were transported approximately 50 miles from a vendor to the animal research facility. Hair samples, which were taken every 40 days, showed that cortisol levels were elevated at 40 and 80 days after transportation, and back to baseline at 120 days. In a follow-up study by the same authors, rabbits underwent the same journey, but were subsequently subjected to surgery, sham surgery, or no surgery (control) 40 days after arrival at the new facility (Peric et al., 2018). Once again, hair cortisol levels were elevated at 40 days after transportation, just before surgery. On day 80 after arrival (and day 40 after surgery), rabbits who had undergone real and sham surgery had significantly higher cortisol levels than control rabbits; specifically, levels in control rabbits were similar to the pre-transport baseline, levels in rabbits who had undergone sham surgery were elevated, but not as much as immediately after transport, and levels in rabbits who had undergone surgery were considerably higher than immediately after transport. By 120 days after arrival (and 80 days after surgery), levels in all three groups were similar to pre-transport levels. Hair cortisol levels reflect values at the time the hair was growing, and the authors hypothesize that the lag time is about 15 days. Accordingly, these two studies suggest that rabbits exhibit a stress response lasting between 26 and 105 days after transport, and that transportation is less stressful than surgery but more stressful than anesthesia.

Figure 2: Stress associated with within-facility transport of rabbits can be reduced by allowing rabbits to hop into a crate lined with a shared Vetbed rather than carrying them by hand (Kinally et al., 2023).

Kinally et al. (2023) described a method to reduce stress when single-housed male New Zealand white rabbits were transported within the facility. To refine the transport procedure, they used a carrying crate lined with Vetbed (a soft plushy material) that was either their own or shared between rabbits who met weekly in a playpen (Figure 2). (While developing this transport method, they found that including a Vetbed was much more effective at enticing rabbits to enter the carrying crate than clicker training and treats.) Rabbits were given up to 3 minutes to voluntarily jump into the carrying crate, otherwise they would be carried by hand. Handlers were always gentle and patient during the 3-minute period, allowing the rabbits to jump in and out on their own without any physical force, to prevent rabbits from forming a negative association with the crate. Rabbits became accustomed to this routine and entered the crate more quickly after the first week, and they entered it within approximately 15 seconds by the end of the study. The authors found that rabbits entered the crate more quickly when the crate contained the shared Vetbed (i.e., one that smelled of other, familiar rabbits) compared to one that smelled only of themselves. Indeed, rabbits with the shared Vetbed would sniff and rub their chin on it during the first few seconds of entering the crate, while rabbits with their own Vetbed somewhat lost interest in the crate after the first 2 weeks. This crate transport method gives rabbits more agency in such handling interactions and may reduce stress related to transport within a facility.

KEY TAKEAWAYS: TRANSPORTATION

- Transporting **rodents** and **rabbits** between facilities is a very stressful event for the animals, causing physiological and behavioral changes that last for days, weeks, or months after moving to a new facility. More research is needed in this area to understand the extent of these impacts in each species and to develop more effective stress-mitigation strategies.
- Stress is induced before, during, and after the trip. Factors causing stress include handling, separation from familiar cagemates, confinement in an unfamiliar container, noise and vibration during transport, disruption of the light:dark cycle, and arrival at an unfamiliar location with unfamiliar people and protocols.
- Thorough planning and care regarding all stages of transportation are necessary to minimize animals' fear and anxiety. Habituation and positive reinforcement training could be especially beneficial before transport. For transfers between facilities, a post-transfer acclimatization period of at least 1–3 weeks should be provided, depending on species and sex.
- During **rodent** transfer, water gel pouches with an X-shaped slit should be hung on the wall of the crate to avoid the gels evaporating or getting buried in bedding and feces.
- Within-facility transfer is also a source of stress for **rodents**; positive reinforcement training before transfer and a minimum post-transfer acclimatization period of 2–4 days is recommended, depending on species. A plastic cart with pneumatic wheels produces the least noise and vibration and is recommended for within-facility transfers.
- In **rabbits**, within-facility transport stress can be minimized by allowing rabbits to hop into a carrying crate lined with a Vetbed shared between rabbits at the facility.

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Conclusion

Our knowledge of animal welfare has grown exponentially in the last few decades, supplying us with more tools than ever to minimize negative experiences for animals in research institutions. Too often, the inertia of tradition is a big hindrance for concerned animal care personnel, veterinarians, and young scientists who wish to change the status quo of rodents and rabbits assigned for biomedical research. We hope that this book can provide those who wish to catalyze change with the information and resources needed to support their efforts.

Approximately half of the studies described in this book pertain to mice; this "overrepresentation" is appropriate, since mice comprise an estimated 30–50% of *all* animals used in research worldwide. This notwithstanding, some species are *under*represented due to a lack of research on refinements to important aspects of their housing and handling. For example, there is little to no research on non-aversive methods for handling any rodent or rabbit species other than mice, and very little research on within- and between-facility transport of any species, despite how prevalent these two procedures are. It is also worth mentioning that, although this book covers a wide range of refinements relevant to rodents and rabbits used in research, there are several key topics that were not covered, such as refinement of euthanasia methods and protocols and refinement of various experimental procedures, including the generation of specific disease models. These procedures are nonetheless incredibly important to the experiences of animals used in research and should continue to be refined wherever possible. We encourage readers to use AWI's Refinement Database to search for current refinements in these areas.

Ultimately, so long as animals continue to be used in research, it is important to make ongoing improvements to their welfare and adapt current practices as new evidence becomes available. This book provides plenty of examples of incremental changes

Figure 1: Dr. Andrea Graham's lab at Princeton has conducted experiments with mice living in circular outdoor enclosures designed to keep mice in and predators out.

that contribute to overall improvements to animal welfare. Moving forward, more substantial changes could bring us closer to providing a good life for rodents and rabbits in research. For example, we hope that the development of remote sensing technologies can encourage the keeping of rodents and rabbits in free-ranging environments where their movements and welfare can be tracked and assessed with little interference (Makowska & Weary, 2019). More naturalistic environments would provide animals maximal choice and control over their lives, in addition to reducing their exposure to many other stressors that exist within an animal facility, representing a win not only for animals but also for science. Indeed, some studies using free-ranging mice—both indoors and outdoors—are already yielding scientific results that are more robust and representative of a "normal" population (Figure 1; e.g., Gaukler et al., 2016; Leung et al., 2018; Zipple et al., 2025).

Until such big changes can be implemented—and in cases where they simply cannot be implemented due to experimental constraints—small changes and a caring attitude can make all the difference in the lives of individual animals. We encourage a mindset of curiosity, compassion, and ongoing commitment to improvement when caring for animals in research environments.

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ABOUT THE AUTHORS

I. Joanna Makowska (PhD) is director of the Animals in Laboratories Program at the Animal Welfare Institute and adjunct professor in Applied Animal Biology at the University of British Columbia (UBC). Joanna completed her doctoral studies at the UBC Animal Welfare Program in 2016. She is particularly interested in the role of choice and agency within naturalistic settings in promoting the welfare of animals under human care. Her research has focused on the impact of housing conditions on rat and mouse welfare, humane methods of euthanasia for rats and mice in laboratories, and how handling methods for captive mice and training methods for companion dogs affect their welfare and relationships with human caregivers. Joanna served more than a decade in total as a member of various animal care–related committees, including the Office of Laboratory Animal Welfare's ICARE faculty, the UBC Animal Care Committee, and the Canadian Council on Animal Care's Standards Committee and Euthanasia Subcommittee.

Anna S. Ratuski (PhD) is a laboratory animal welfare research fellow at Stanford University, working on the Department of Comparative Medicine's Beyond3Rs initiative. Anna completed her doctoral studies at the UBC Animal Welfare Program in 2023. Her research interests include testing practical strategies to improve the lives of animals in captivity and studying how animal behavior and biology are affected by their environment. Her doctoral research focused on environmental enrichment approaches for rats and mice housed in laboratories. Her other research areas include humane methods of euthanasia for rats and mice in laboratories, how breeding environment affects mouse welfare and reproductive outcomes, and abnormal behavior in mice. She was inspired to pursue animal welfare research in 2017 after working as an animal care technician in a research facility. Anna has also served on the UBC Animal Care Committee and previously taught an undergraduate course on welfare and ethics of using animals in science.

ABOUT THE ANIMAL WELFARE INSTITUTE

The Animal Welfare Institute is a nonprofit charitable organization founded in 1951. AWI is dedicated to alleviating animal suffering caused by people and seeks to improve the welfare of animals everywhere: in agriculture, in commerce, in our homes and communities, in research, and in the wild. To achieve its mission, AWI works in eight programmatic areas: Animals in Laboratories, Companion Animals, Equines, Farmed Animals, Humane Education, Marine Wildlife, Terrestrial Wildlife, and Government Affairs.

AWI's Animals in Laboratories Program works to foster the development and implementation of science-based housing and handling refinements that reduce pain and distress and provide opportunities for species-appropriate mental and physical stimulation and social interaction. To this end, AWI has created many resources geared toward individuals who work with animals in laboratories:

- → AWI's long-running online Laboratory Animal Refinement & Enrichment Forum (LAREF) facilitates meaningful discussion and exchange of ideas among members of the research animal care community concerning ways to improve the conditions under which animals in laboratories are housed and handled.
- → AWI also provides funding to laboratory personnel to conduct research focused on improving the housing and handling of animals in research (via the Refinement Research Award) and to implement better practices (via the Implementing Refinement Grant).
- → Additionally, AWI creates and distributes publications—such as this book—at no cost to laboratory personnel, and curates a free, online Refinement Database of scientific articles and books on topics related to improving or safeguarding the welfare of animals in research.
- → Finally, AWI conducts original research and data analyses to expand scientific understanding of the welfare needs of animals in research, and campaigns to help shape laws and policies to improve the lives of animals in research.

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