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Review Article

A Review of the Effects of Some Extrinsic Factors on Mice Used in Research

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Animals have been used in research for over 2,000 y. From very crude experiments conducted by ancient scholars, animal research, as a science, was refined over hundreds of years to what we know it as today. However, the housing conditions of animals used for research did not improve significantly until less than 100 years ago when guidelines for housing research animals were first published. In addition, it was not until relatively recently that some extrinsic factors were recognized as a research variable, even when animals were housed under recommended guidelines. For example, temperature, humidity, light, noise, vibration, diet, water, caging, bedding, etc., can all potentially affect research using mice, contributing the inability of others to reproduce published findings. Consequently, these external factors should be carefully considered in the design, planning, and execution of animal experiments. In addition, as recommended by others, the housing and husbandry conditions of the animals should be described in detail in publications resulting from animal research to improve study reproducibility. Here, we briefly review some common, and less common, external factors that affect research in one of the most popular animal models, the mouse.

Abbreviations and Acronyms: BPA, bisphenol A; EE, environmental enrichment; HSP, heat shock protein; QAC, quaternary ammonium compound; RH, relative humidity

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Introduction

Animals have been used as research subjects for over 2,000 y. Between the fourth century BC and the second century AD, Greek-speaking scholars such as Aristotle, Herophilus, Erasistratus, and Galen began the systematic dissection of animals and comparative investigation of their anatomies.[38] From those crude beginnings, and after hundreds of years and refinements in the conduct of experiments, major advances have been achieved in our understanding of biology and medicine. However, the housing conditions of the animals that were used for the necessary research did not improve significantly until less than 100 years ago when guidelines for housing research animals became available. In 1963, the Animal Care Panel published the first edition of the *Guide for Laboratory Animal Facilities and Care* with recommendations for the care and housing of mice and other species used in biomedical research.[7] The effect of the environment as an important research variable was not recognized until the 1970s.[70,231,238] Much has been learned in the last 50 y about the husbandry requirements and care of research animals. However, much room for improvement remains. Extrinsic factors (i.e., extrinsic environmental factors) that are part of management practices can have significant effects in the animal's physiology and, consequently, on the data being collected from these animals. These extrinsic factors may vary among research facilities, within the same facility, within the same animal room, and even within the same cage, with potentially significant effects on data that contribute to study irreproducibility. In 2014, an article titled "NIH plans to enhance reproducibility" expressed concerns about animal studies, saying "Preclinical research, especially work that uses animal models, seems to be the area that is currently most susceptible to reproducibility issues. Many of these failures have simple and practical explanations: different animal strains, different lab environments or subtle changes in protocol."[35] That paragraph also recognizes "different lab environments" as a possible cause for study irreproducibility. In 2020, the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines added housing and husbandry information to the list of information that should be provided when reporting studies that used live animals, as follows: "Provide details of housing and husbandry conditions, including any environmental enrichment."[189] However, this recommendation is not currently included on the ARRIVE Guidelines "Essential List". In 2022 the NIH hosted a workshop to discuss external factors affecting rigor and reproducibility of animal-based research. This workshop generated 3 reports, one of them focused exclusively on rodents. Various extrinsic factors were identified for consideration in research, including personnel, caging type, housing density, ambient temperature, food and water, bedding, enrichment, cage-change frequency, species-specific measures of behavior, the microbiome, lighting, vibration, and air.[173] A commonly expressed view at this workshop was that extrinsic factors in animal research are unlikely to be standardized across institutions and laboratories and that systematic variation in animal studies (e.g., performing the same study on several different mice strains or in different physical locations or at different environmental temperatures), along with the use of completely randomized and randomized block

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design, may improve reproducibility between experiments and better represent the biologic systems of humans.[63,173,201] A recent meta-analysis suggests that conventional rodent housing may be sufficiently distressing to compromise rodent health, causing the rodents to be "cold, rotund, abnormal, male-biased, poorly surviving, enclosed and distressed."[26] These possibilities raise questions about the validity and use of the data they generate. Apart from the well-recognized factors that affect animal research, we will discuss other less obvious factors in this manuscript. The animal research community should be aware of these subtle, but important, external factors and how they can impact study reproducibility because extrinsic factors (i.e., diet, ambient temperature, light) may alter intrinsic factors (i.e., microbiome, gene expression, physiology, immunity, circadian rhythm), thus supporting the importance of detailed reporting of the housing condition that the animals had experienced (Table 1). The purpose of this review is to discuss with and alert the animal research community about how currently recommended housing practices for mice may influence study results and why housing and husbandry practices should be described in detail in publications resulting from animal research. Some of the factors discussed have been known for years, but little attention has been given to others that either are relatively new and still not widely recognized by the animal research community or require further studies to corroborate initial studies.

Temperature

An early publication by the Jackson Memorial Laboratory in 1941 recommended housing mice at "approximately 72 °F" (22 °C) "at all times" in cages made out of wood to protect them from drafts, with woodchips and sawdust as bedding, and with a small piece of cotton as nesting material.[203] Later, in 1957, the Institute for Laboratory Animal Research published temperature guidelines for housing research mice, recommending mice room temperatures be maintained between 21.1 and 26.6 °C.[112] Since then, this guideline has not changed significantly, with the most recent version of the *Guide for the Care and Use of Laboratory Animals* recommending housing mice at slight lower temperatures, between 20 and 26 °C, with minimal fluctuation near the middle of these ranges; this midrange would be 23 °C, which is well below the thermoneutral zone of mice (26 to 34 °C).[113] Following this guideline means that research mice are chronically subjected to cold stress, which is particularly critical for neonates, weanlings, singly housed mice, nude mice, sick mice, and mice housed in mechanically ventilated cages.[45,46,237]

Cold stress is perhaps one of the most important extrinsic environmental conditions affecting laboratory mice. Numerous studies have shown that chronic cold stress profoundly affects mouse physiology, requiring metabolic adaptations that could interfere with modeling of human homeostasis and disease. Adaptation to chronic cold stress involves marked changes in glucocorticoid production and activation of the sympathetic nervous system, which in turn affect immune responses to infectious agents, cancer, drugs, affects the gut microbiome, breeding, growth, and behavioral studies, to name a few.[30,46,88,108-111,115,155,196,232,237-239]

Several examples illustrate these effects. The survival of male C57BL/6 mice housed in climate-controlled chambers with contact bedding and nesting material at 30 °C was 78% after cecal ligation and puncture as compared with 40% survival of mice housed at 22 °C.[30] Another study assessed energy expenditure

Table 1. Summary of some external factors' potential effects on mice

Factor	Potential Effect on
Temperature	Infectious disease, immune system, energy expenditure, brown adipose tissue, thermogenesis, tumor metabolism, bone metabolism, core temperature, motor activity, gut microbiome, embryo yield, and heart rate
Relative humidity	Cornea's integrity, aqueous tear production, $CD4^+$ cell infiltration in lacrimal glands, embryo production, time to first estrus, and gut microbiome
Air quality	Anxiety, pain, depression, immune system, cardiac allograft survival, lipolysis, thermogenesis, blood pressure, food intake, behavioral and neural responses, stress responses, abortion, number of offspring born, plasma corticosterone, fecal boli, core body temperature, and hematology
Light	Disease susceptibility, cancer incidence, retinal abnormalities, acute phase response, inflammation and innate immune response in skin and brain, sleep cycle, and circadian rhythm
Noise and vibration	Circadian rhythm, cardiovascular and immune systems, behavior, blastocyst production, embryo resorption, litter size, and ultrasonic communication
Caging material	Body growth and abdominal adiposity, timing to puberty, estrogen receptor expression patterns in vagina, mammary gland tissue growth, prostate weight, epididymal weight, daily sperm production, and meiotic effects
Bedding	Liver microsomes, respiratory system, immune system, gut microbiome, body/organ weight, ghrelin and glucose plasma levels, energy content, and disease model phenotype
Diet	Gut microbiome, α and β diversity, inflammatory or invasive microbiota, intestinal permeability, proinflammatory mediators, immune cell response, influenza infection modulation, colorectal tumorigenesis, inflammatory bowel disease, obesity, dermatitis, relative uterus size, embryo yield, and behavior
Water	Gut microbiome, motor behavior, neuropathology, diabetes, intestinal mucosal inflammatory cells and expression of cytokines and transcription factors, and disease model phenotype
Metal ear tag	Auricular chondritis and autoimmunity
Disinfectants	Sperm concentration and motility, time to first litter, pregnancy intervals, pups per litter, morbidity in near term dams, neural tube defects in fetuses, genotoxicity, allergic responses, immune system, pulmonary disease, and gut microbiome
Enrichment	Pup weigh and survival to weaning age, behavior, immune system, antitumor immunity, and corticosterone plasma levels
Group housing	Immune system, body weight, food intake, adiposity, glycemic control, insulin signaling, adipose tissue inflammatory response, gene expression, infectious and tumor studies, body temperature, behavior, energy expenditure, bone metabolism, sperm count and motility, gonad development, time in estrus, gut microbiome, and disease model phenotype
Human handling	Corticosterone, glucose, growth hormone and prolactin plasma levels, heart rate, blood pressure, urination/defecation, behavior, and disease model phenotype

in C57BL/6J and Crl:NU-Foxn1(nu) nude mice that were group-housed in individually ventilated cages (5 mice per cage, 60 air changes hour) with corncob bedding and cotton nesting material at low (21 °C), intermediate (26 °C), and high (31 °C) temperatures.[45] Energy expenditure was significantly higher in mice housed at low temperature as compared with those housed at intermediate and high temperatures and was associated with a shift in metabolism toward glucose utilization. Brown adipose tissue showed significant activation at low and intermediate temperatures as compared with 31 °C in both C57BL/6J and Crl:NU-Foxn1(nu) nude mice, with Crl:NU-Foxn1(nu) mice experiencing greater cold stress than did C57BL/6J mice.[45] In another study, male CB17/lcr-Prkdcscid/lcrlcoCrl (Prdkcscid) and Crl: Nu-Foxn1Nu (Nu-Foxn1Nu) mice (age, 10 wk) were singly housed in individually ventilated cages, with or without shelters, and in static cages with corncob bedding and maintained at 20 to 21 °C.[46] Those housed in individually ventilated cages had histologic signs of cold stress (greater chronic activation of brown adipose tissue) and significantly more nonshivering thermogenesis, smaller subcutaneous epidermoid carcinoma tumors, lower tumor metabolism, and larger adrenal weights than did mice in static cages.[46] The shelters partially protected mice from cold stress. The authors recommend a slightly higher macroenvironmental temperature around 24 °C along with enriching individually ventilated cages with nesting material or translucent shelters.[46] Randomly bred ICR/Alb mice, housed in shoe-box style polypropylene cages with wood shavings as bedding and a cotton square as nesting material were subjected to different temperatures and humidity; the study found mice maintained at 25 °C attained puberty earlier than mice maintained at 20 °C or lower temperatures.[55] Another study found that the type of bedding material affects thermoregulatory stability, core temperature, and motor activity in female CD-1 mice housed in groups of 4 in standard static cages at ambient temperature of 23.5 °C.[79] Mice housed with a deep layer of wood shavings maintained significantly higher core temperatures during the day as compared with mice housed with a thin layer of wood shavings and β chips.[79] During the night, core temperature and motor activity were high in all group, with no effect of bedding type. The author concluded that housing mice on a deep layer of wood shavings or comparable materials is conducive to burrowing, which reduces heat loss.[79] In a study of female 6-wk-old BALB/cAnNcr that were housed 5 per cage in microisolation cages at either 22 to 23 °C or the thermoneutral temperature of 30 to 31 °C for 6 wk, the gut microbiome of mice housed at 22 to 23 °C had an enrichment of members of the family Lachnospiraceae, indicating that adrenergic stress and need for energy to support thermogenesis modulates the gut microbiome.[110] In another study, genetically modified mouse lines and wild-type mice with corresponding genetic backgrounds (C57BL/6; NMRI) were housed in individually ventilated caging systems and barrier facilities at 22, 24, and 26 °C; embryo yield was significantly higher when donor mice were fed a phytoestrogen-poor diet at 24 °C.[196] C57BL/6J male mice 3 to 6 mo old, singly housed at 23 ± 1 °C and Sani-Chips bedding at a depth of approximately 1 cm in conventional open-topped cages with wire lids and without microisolation lids showed tachycardia that was presumably related to cold stress.[31] A recent study found small differences in temperature (4 °C) or heat loss (individual compared with group housing with cotton squares) influence bone growth in 5-wk-old female C57BL/6J mice.[211] In this study, mice that were individually housed at 22 °C had greater body weight and femur size, but dramatically lower cancellous bone volume fraction in the distal femur metaphysis. The cancellous bone loss was attenuated but not prevented in mice housed individually at 26 °C or in group-housed mice at 22 °C.[211] In another study, 12-wk-old male C57BL/6NCrl mice were housed individually at room temperatures of 30, 21, or 4 °C with a cardboard shelter and wood-wool nesting material.[64] Based on high time-resolution calorimetry analysis, mice housed at the thermoneutral 30 °C temperature displayed mean diurnal energy expenditure rates that were 1.8 times higher than basal metabolism, closely resembling the ratio observed in humans under normal living conditions. The authors concluded that at any temperature below thermoneutrality, mouse metabolism exceeded the human equivalent, which has important implications for study translatability.[64]

Mice may be kept at temperatures that are below thermoneutrality because of concern for heat stress. One study found that B6D2F1 and C57BL/6N dams had fewer weaned pups when kept at 30 °C than did dams kept at 22 °C, with no differences in levels of fecal corticosterone metabolites in dams kept at either temperature.[133] In another study, 7-wk-old male C57BL/6 mice were housed 4 per cage at 22 °C with food available ad libitum, at 35°C for 5 d with food available ad libitum, or at 22 °C with 5 d of food restriction.[167] Mice kept at 35°C for 5 d had significantly higher leptin and adiponectin signaling in white adipose tissue, muscle, and liver as compared with the other groups. Chronic heat exposure that was independent of food intake appeared to be responsible for improving insulin sensitivity and glucose uptake in peripheral tissues, probably mediated by adipokines. The authors concluded that moderate chronic heat exposure could be a potential therapeutic treatment for disorders associated with insulin resistance.[167] A study to assess the effects of chronic heat stress on humoral and cellular responses of DNA vaccination showed that 6- to 8-wk-old female Balb/c mice exposed to a temperature of 38 °C for 2 h per day, for 35 d had poorer responses to the vaccination, particularly impaired the cell-mediated responses, as compared with mice maintained at 24 °C for the same period of time.[102] Chronic heat stress significantly lowered levels of IgG2a, T cell proliferation, expression of interferon-γ in $CD4^+$ and $CD8^+$ cells, and cytotoxic T-lymphocyte responses.[102] Another study found that chronic heat stress (37 and 40 °C) adversely affected testicular structure and spermatogenesis and caused inflammation leading to testicular interstitial fibrosis in 8- to 10-wk-old male Swiss mice as compared with a control group housed at 25 °C.[175] Short, repeated exposure of male C57/BL6J mice (aged 8 to 16 wk) to a high ambient temperature (40 °C and 40% relative humidity [RH]) induced acute kidney injury that was ameliorated by previous repeated sessions of exposure to mild heat (38 °C and 40% RH).[81] In another study, male C57BL/6 mice (12 wk of age) were placed in a temperature-controlled chamber at 43 °C and 60% ± 10% humidity for 15 min a day for 7, 14, 21, and 42 d.[179] The study showed that repeated heat exposure augmented oxidative stress, endoplasmic reticulum stress, and apoptosis pathways in the cerebellum.[179] Three-week-old female ZCK mice that were continually exposed to 25 °C and 50% humidity for 1, 3, or 6 wk or to daily 9- min exposure to 39 °C at 50% humidity for 1, 3, or 6 wk. The repeated heat exposure reduced whole-body and ovarian growth but had little effect on the ovarian index (ovary wet weight in mg/body weight in g × 100%, ±SE); acute heat exposure did not alter whole-body or ovarian weight. The authors hypothesized that chronic and acute exposure to heat impaired ovary function by causing the dysfunction of granular cells.[16] In another study, adult male and female CD-1 mice were housed for 5 d at 37 °C, and core temperature, heart rate, and activity were monitored telemetrically; heat shock proteins

(HSPs) were measured in brain and lung by western blotting.[24] The study showed that female mice exposed to this condition maintained a core temperature that was 1.2 °C lower than in age-matched males (38.3 ± 0.6 and 39.5 ± 0.7 °C, respectively), experienced less weight loss (1.5 ± 0.5 compared with 4.5 ± 2.0 g), and had greater survival (16/16 compared with 13/21).[24] After 5 d of moderate heat exposure, HSP72 is increased 2.1-fold in brain and lung and 5-fold in male mice as compared with 1.3- and 1.5-fold in female mice.[24] In another study, female BALB/c mice, 8- to 10-wk-old, were placed in an incubator for 21 d and exposed to a temperature of 38 ± 1 °C for 4 h; mice in the control group were kept at 24 ± 1 °C to simulate room temperature.[119] The heat-exposed mice showed a reduced local immune response in the respiratory tract, with reductions in the number of pulmonary alveolar macrophages and more lesions in the nasal mucosa, trachea, and lungs as compared with mice kept at 24 °C. Chronic heat exposure retarded dendritic cell maturation and reduced the mRNA levels of IL-6 and IFN-β. After infection with H5N1 virus, the mortality rate and viral load in the lungs of the chronically heat-exposed mice were higher than those of the control group. The authors concluded that chronic heat exposure can suppress local and innate immunity in the respiratory tract and consequently promote the virulence in H5N1-infected mice.[119] These examples show that housing mice above their thermoneutral zone can have deleterious effects.

Some studies suggest mice, like humans, may adapt to living in calid environments.[209] For example, male CD-1 mice weighing 30 to 35 g housed in standard cages with bedding and a plastic igloo were continuously exposed to mild hyperthermia (ambient temperature approximately 37 °C that caused approximately 2 °C increase in core temperature) for 5 d and were then exposed to a thermal stress (42 °C ambient temperature for 40 min) and compared with control mice that had no previous acclimation to mild hyperthermia before exposure to thermal stress.[209] The acclimated mice showed slower warming during thermal stress, more rapid cooling during recovery, greater activity during thermal stress, and some of the features of acquired thermal tolerance, including higher baseline expression of HSPsp72 and HSP90 in lung, heart, spleen, liver, and brain, and a blunted incremental increase in HSP72 expression after acute thermal stress. The authors suggest that continuous 5-d exposure of CD-1 mice to mild hyperthermia induces a state that resembles the physiologic and cellular responses of human acclimation to heat.[209]

An extensive review of the effects of temperature, humidity, air changes, and caging on different species of research animals included recommended temperatures that required minimal metabolic adaptation.[237] The recommended temperature for minimum metabolic adaptation of a single mouse in a static cage with free air exchange is 31 to 34 °C for a hairless mouse and 26 to 31 °C for an albino mouse.[237] These temperature ranges will decrease if the cage system has limited air exchange and contains multiple mice. The ranges may also require extension to provide warmer conditions for mice with disturbed thermoregulation due to diminished heat production due to sickness or recovery from anesthesia or increased heat loss due to, for example, mechanically ventilated cages. In the latter case, thermoneutral conditions can be reestablished by increasing the ambient temperature. Keeping mice at 20 to 22 °C will cause mice to undergo cold adaptation.[237-239] However, these recommendations may not be considered despite the potential effects of chronic cold exposure on mice and the research they are used for. The *Guide for the Care and Use of Laboratory Animals* currently states "Animals should [rather than must] be housed within temperature and humidity ranges appropriate for the species, to which they can adapt with minimal stress and physiologic alteration".[113] The Guide recommends a macroenvironment temperature of between 20 and 26 °C for mice, with minimal fluctuation near the middle of these ranges (i.e., 23 °C); however, many institutions keep rodent rooms around 21 to 22 °C.[45,144,221] The Guide also says, "animals should be provided with adequate resources for thermoregulation (nesting material, shelter) to avoid cold stress." Again, the word "should" is used. The Guide does not mandate the use of nesting material, shelters, or other sources to protect mice from cold stress, leaving to the individual institutions or investigators the decision of whether steps will be taken to prevent cold stress in mice.[88] The temperature in an individually ventilated cage is usually 1 or 1.5 °C warmer than the room temperature if the cage holds 5 mice that have bedding and nesting material, although the use of nesting material and shelters attenuates but does not completely counteract the effects of low intracage temperature.[46,73-75] In addition, some inbred and genetically modified mice strains do not build or poorly build nests, contributing to the potential need to protect them from cold stress.[6,53,71,151] One group reported that up to 10 g of nesting material may be needed for mice to build a nest that will alleviate thermal distress under temperatures of 20 to 24 °C.[73-75] Commonly used commercially available mice nesting material such as cotton squares weigh between 2.2 and 2.6 g, meaning that depending on the number of mice in a cage, 4 or more cotton squares may be required to allow mice to build a nest that will provide thermal regulation. Institutions that report the use of cotton squares usually provide one per cage, which is not sufficient for allowing mice to build nests that will efficiently mitigate cold exposure.[45,71,80,225,244,245] The microenvironmental cage temperature is further influenced by the caging system used and husbandry components that include bedding substrates, enrichment devices and materials, number and age of the mice, cage density, light exposure, and the mouse's phenotype.[88] As described above, cold stress in research mice may go beyond what could be defined as "minimal stress and physiologic alteration". The most common reason for keeping rodent facilities at low temperatures is to provide human comfort and to avoid the high cost of maintaining facilities at relatively warm temperatures.[144] However, the cost savings obtained by keeping rodent facilities at relatively cold temperatures is likely negligible relative to the costs of conducting preclinical research in a potentially compromised animal and generating data that may not be reproducible or translational.[67] Numerous studies indicate that currently recommended environmental temperatures for mice should be revised to move closer to the species-specific lower limit of the thermoneutral zone and to provide enough bedding and nesting material for behavioral thermoregulation.[64,115,127,155,237-239]

Relative Humidity

Current guidelines recommend housing mice at a RH between 30% and 70%.[113] Extremes of RH (<30% or >70%) are known to significantly affect animal physiology and metabolism and predispose to health problems.[48,125,139,144] In general, a RH between 30% and 70% is considered adequate for research mice, but the effects of subtle shifts in RH may not be easily detected on routine health checks and may have or not affect the study and/or animal well-being. For example, eye desiccation stress can occur in more C57BL/6J mice housed in mechanically ventilated cages with 43.5% RH than occur in mice housed at 53.9% RH.[259] At 43.5% RH, aqueous tear production is reduced, and corneal permeability and irregularity increase.[259] Histologic

examination of the low-humidity mice revealed desquamation of corneal basal epithelial cells, reduced goblet cell density in conjunctival epithelia, elevated $CD4^+$ cell infiltration of lacrimal glands, and displacement of keratin-14-positive limbal epithelial stem cells on the ocular surface.[259] Some studies suggest that a very narrow range of RH is best for embryo production in C57BL/6N, FVB/N, NMRI, 129 S6, and a few other mice strains, with decreases in production occurring when the RH decreases below 40% or increases above 45%.[51,213] On the other hand, ICR/Alb female mice housed at 60% RH attained first estrus significantly earlier than did mice that were housed at 30% RH.[55] To further complicate effects of RH, a recent study found short-term exposure to high RH alters the gut microbiota of 8-wk-old male BALB/c mice.[253] These effects may or not be important depending on the type of research being conducted. Variation in RH could contribute to variability in vision studies or may be significant to investigators trying to rescue a line that breeds poorly. Clearly, further studies are required to determine possible subtle effects of RH on mice.

Air Quality

Depending on geographic location and proximity to heavily industrialized areas, air quality can vary markedly. Most animal facility HVAC systems use external air and may recirculate part of the internal air to save energy. Pollutants in external air, apart from those that can be eliminated by mechanical filtration, are difficult to control and may or not have an affect in mice. One study found that aged AKR/J mice had more cardiac alterations when exposed to real-world air pollution at concentrations that mimic human exposure as compared with AKR/J mice exposed to filtered air.[197] The effects of common pollutants that are generated by the mice themselves or from their waste (i.e., CO_2, ammonia, and allergens) are well known, and husbandry practices have been developed to limit their effects. Other less studied organic volatile compounds may be ubiquitously present in an animal facility and may affect both the mice and the research. Personnel wearing perfumes, fragrances, or deodorants in the animal facility cause confounds in study results. Essential oils, commonly used in colognes, perfumes, and fragrances, are used in aromatherapy because of their purported medical properties. For example, aromatherapy improves cognitive dysfunction in male SAMP8/TaSlc mice, has antianxiety effects in male Swiss mice, has sedative effects and reduces hyperalgesia in female Swiss mice, reduces pain and stress-induced immunosuppression in male ddY, male CD-1 Swiss, female OF1, and female C57BL/6J mice, reduces depression-like behaviors in male ICR mice, alters the immune response of female BALB/c and C57BL/6J mice, and prolongs cardiac allograft survival in male CBA mice.[25,52,54,68,69,120,142,180,187,215,234] Olfactory stimulation with the scent of grapefruit oil increases the activity of sympathetic nerves that innervate white and brown adipose tissues, the adrenal glands, and the kidneys, and decreases the activity of the gastric vagal nerve in rats and 6-wk-old-male Jcl:ICR mice, resulting in increased lipolysis, thermogenesis, and blood pressure and a decrease in food intake.[172] In contrast, the olfactory stimulation of lavender oil decreases blood pressure, thermogenesis, and body temperature by reducing brown adipose tissue-sympathetic nerve activity in rats and Jcl:ICR mice.[172] Behavioral and neural responses to the irritant allyl isothiocyanate, present in cruciferous vegetables, were effectively mitigated by olfactory costimulation with phenlyethyl alcohol or lavender oil in male C57BL/6N mice.[186]

Mice, like other animals, seem to discriminate certain odors emitted by humans that are related to different emotions. The physiologic processes associated with an acute psychologic stress response in humans produce changes in the volatile organic compounds emanating from breath and/or sweat and can be detected by dogs.[42,248] Mice and other animals appear to recognize chemical signals associated with stress in humans and other animals; this recognition results in physiologic and behavioral reactions that indicate induced stress in response to the stress odor of humans and other animals.[2,27,42,50,77,116,254] For example, 6- to 8-wk-old male C57BL/6J mice defecated more in the presence of the stress odor than the nonstress odor in sweat.[50] Cat urine odor increased the proportion of miscarriages in female CrlFcen:CF1 mice, whereas the odor of unfamiliar male mice reduced the mean number of pups born per female.[2] A study using 4-wk-old male C57BL/6J mice found the injection of LPS altered the odors that mice used for inter- and intrasexual communication; the odors of healthy mice living with LPS injected mice more closely resembled the odors of sick as compared with healthy mice.[77] The authors suggest that the odors of sick (LPS-injected) cage mates induced physiologic changes in the healthy mice, and that these physiologic changes may resembled the alterations induced by LPS.[77] Another study reported that axillary secretions of men but not of women likely triggered stress-induced analgesia in CD-1 and C57BL/6J mice.[220] Baseline pain latencies or thresholds in 3 different acute pain assays were significantly higher when testing was performed by a man rather than a woman, indicating a possible analgesic effect associated with men.[220] In addition, exposure to men in the absence of pain increased plasma corticosterone levels and fecal boli; core body temperature increased more rapidly when male researchers were taking rectal temperatures as compared with females. The authors concluded that, although brief, stress caused by male researchers may exert a common confound in animal research.[220] This study was conducted by directly exposing mice to odors that they may not encounter under normal conditions in an animal facility. However, these organic volatile compounds may persist in the environment even after the person leaves the room, potentially exposing mice to its disruptive effects. For this reason, personnel should avoid wearing perfumes, fragrances, or deodorants in the animal facility, and the potential effect of male experimenters should be considered when designing studies. Another study found that oxygen in the air in individually ventilated cages was 2.5% less than in the ambient environment when male C57BL/6J mice were housed 4 per cage, resulting in chronic low-grade hypoxia and hematologic and behavioral changes.[254]

Light

Light is another important extrinsic environmental condition. A recent excellent review of vivarium lighting discussed how different types of light, intensity, duration, wavelength, caging material, and cage position in room and rack affect light exposure and consequently metabolism and susceptibility to disease.[43] The review also discusses the effect of light contamination on cancer incidence and other types of studies.[43,44] An early study found female BALB/c mice develop retinal atrophy due to exposure to vivarium lighting.[83] The study reported that at 33 mo of age, retinal atrophy affected 30% of the mice housed on the top shelf, followed by 12% on the next shelf, and fewer than 1% of those housed on lower levels.[83] BALB/cJ mice housed under normal vivarium lighting conditions can exhibit profound retinal abnormalities, including retinal infoldings, autofluorescent inflammatory cells, and photoreceptor degeneration.[17] Advanced age and top row illuminance results in significant photoreceptor cell loss as demonstrated by

decreased thickness of the outer nuclear layer. These changes are preceded by retinal infoldings and the presence of autofluorescent inflammatory cells in the outer retina. The authors of the review suggest that these changes are early indicators of light toxicity in BALB/cJ mice and recommend vivarium lighting should provide approximately 30 lx about 1 m above floor level, which provides enough light for investigators and husbandry staff to perform their duties.[17] They further indicate this illuminance level should translate into external and internal cage illumination intensities of approximately 10 to 15 and approximately 0.5 to 1.5 lx, respectively, and that prolonged exposure to other sources of light such as cage change stations or safety cabinets should be avoided.[17] A more recent study found that fluorescent light induced transcriptional changes of the acute phase response signaling pathway and modulated inflammation and innate immune responses in skin and brain of hairless mice.[22] The authors of that study suggested cellular perception of oxidative stress promotes the induction of primary upstream regulators IL-1B and TNF, with skin and brain developing inflammatory and immune responses.[22] Most research rodents are nocturnal; the light phase in an animal facility corresponds to their resting phase. Most of the husbandry and research work (health checks, animal treatments, cage changes, experimental manipulation, animal transfers, rack movement, nearby use of autoclaves, cage wash, etc.) is performed during the light phase. The constant activity near or in the animal rooms may disrupt the sleep cycle of mice potentially affecting the circadian rhythm.[147] Numerous studies indicate that most biologic functions are linked to circadian rhythms, and circadian disruption profoundly affect both the animal's physiology and research data.[5,32-34,43,59,85,103,150,153,235]

Mice and rats are often thought to be functionally blind under red light, based on the fact that they are dichromats that possess UV and green cones but not red cones. However, a recent study found that rats retain visual capacity under red light; the inability to see the color red does not necessarily rule out vision based on red light absorption.[177] Reverse light cycles with low-pressure sodium lights, whose wavelength spectrum (589 nm) is not visible to rodents, but can be seen by humans, should be used in rooms housing rodents so routine husbandry activities may be performed during the dark phase of the light cycle, when rodents are active.[160]

Noise and Vibration

Noise and vibration generated during normal facility operation hours can disrupt rodent circadian rhythms.[9,14,188,200] Noise and vibration can alter reproduction, cardiovascular function, and immune function in mice and may elicit behavioral reactions consistent with a fear response.[9,72,114,170,255] Noise from ventilated racks has been linked to decreased blastocyst production in female C57BL/6 mice.[255] Auditory stress also causes higher rates of resorption of C57BL/6 mouse embryos and reduction of litter size.[114] Ultrasonic noise has been less studied. However, we know that mice can hear ultrasonic noises that are beyond the hearing range of humans and may not be measured in animal facilities.[91,171] Mice use ultrasonic vocalization to communicate, including courtship, and environmental ultrasonic noise may interfere with this communication.[91,171,188] The sound of metal impacting metal impact, as commonly occurs in animal facility operations, can reach intensities that allow to be easily audible to mice in cages on individually ventilated racks, potentially exposing the mice to a substantial amount of noise across a wide range of frequencies that they can hearr.[180]

Caging Material

Polycarbonate and polysulfone cages and water bottles made of these materials are commonly used in animal research facilities. Caging material has long been recognized as affecting study results.[49] Several studies have shown that autoclaving of these cages promotes the leaching of chemicals, principally bisphenol A (BPA), a monomer with estrogenic activity.[99,106,132,138] The monomers hydrolyze and leach from these cages and bottles under high heat and alkaline conditions, and the amount of leaching increases as a function of use of the item.[138] Exposure to low, environmentally relevant levels of BPA have a significant effect on reproductive function in female and male rodents (accelerated growth and timing of puberty, altered estrogen receptor expression patterns in the vagina, increased proliferation of mammary tissue, increased prostate weight, decreased epididymal weight, and decreased daily sperm production).[174] Considerable amounts of BPA (approximately 0.15 µg/L) are leached from polycarbonate bottles during the first 24 h of storage after being washed using an alkaline base detergent in an automated cage washing system, allowed to air dry, and then filled with water.[95] Bisphenol F is increasingly being used as a substitute for BPA in the manufacturing of polycarbonate cages and water bottles and consumer products. Exposure of rats to bisphenol F showed significantly increased body growth and abdominal adiposity, which are risk factors for cardiometabolic disease.[233] BPA and its substitutes also affect reproductive organs, and their effects on aquatic species is a global concern.[41] Currently, few data are available on chemical damage and BPA release from polysulfone and polyetherimide, the 2 other common used thermoplastics that have a BPA component and are used in caging. Although these polymers are considered to be stable in comparison with polycarbonate, the finding of passive migration of small amounts of BPA from new polysulfone caging at room temperature in a neutral solution suggests that further research is warranted.[99] One study found housing 129S1/SvimJ and C57BL/6J mice in damaged polysulfone cages exposed them to bisphenol S that had harmful effects similar to those of BPA.[80,96]

Bisphenol analogs released from damaged polysulfone cages elicit meiotic effects in 129S1/SvimJ and C57BL/6J mice, and these persist in males for several generations, suggesting that bisphenols as a class should be considered germline toxicants.[96] Meiosis is both a sensitive indicator of environmental toxins and, because recombination directly affects the amount of genetic diversity in a population, it is also considered an evolutionary driver.[96] Exposure to common replacement bisphenols induces germline effects in both sexes of 129S1/SvimJ and C57BL/6J mice and may thereby affect subsequent generations.[96] Thus, exposure to chemicals that influence recombination are cause for concern. Polypropylene cages and glass water bottles do not leach BPA.

Bedding

Softwood beddings has long been known to induce drug-metabolizing enzymes in liver microsomes of mice and rats.[230] Softwood bedding comprised of either red cedar, white pine, or ponderosa pine induced 3 drug-metabolizing enzymes in liver microsomes of 10 inbred and 2 outbred strains of adult male and female mice (mice strains/stock not defined). The induction was reversed when the mice were removed from the softwood bedding and placed on hardwood bedding comprised of mixture of beech, birch, and maple.[230] Hardwood and corncob beddings, which are commonly used beddings in bedding for research rodents, contain high concentrations of

endotoxins.[60,159,206,246] The potential effect of endotoxin exposure from bedding should be considered when conducting studies that involve LPS or assess respiratory or immunologic end points.[28,60,104,206,210,246] In addition, dust levels can be higher in hardwood beddings.[104,246] Studies in rodents show that repeated airway exposure to wood dust can elicit lung inflammation, which is accompanied by induction of several proinflammatory cytokines and chemokines.[104]

Corncob bedding contains high fungal spore loads and are a risk for fungal infections in immune compromised rodents because the fungal spores remain in corncob bedding even after autoclaving.[206,210] Corncob bedding also contains estrogenic compounds.[224] A study in mice of both sexes, spanning a wide variety of ages, genotypes, and genetic backgrounds, including CD1, Balb/c, C57BL/6, and 129, found that paper beddings contain significantly lower levels of endotoxins and dust and are preferable when endotoxins and dust are a concern, such as during immunologic, respiratory, or inhalation studies.[185,224] In addition, male C57BL/6J and C57BL/6NRj and female Crl:CD1 (ICR) and C57BL/6NCrl mice on calorie restriction consume bedding and feces, which affects body and organ weight, ghrelin and glucose levels in plasma, energy content in gastrointestinal tract, gut microbiota, cecal content metabolites, and rodent phenotypes.[4,58,84,134]

The type of bedding used to house C57BL/6 mice can markedly affect the dynamic range of mechanical and thermal behavioral tests in normal mice and those with tissue injury, with aspen wood chip bedding producing the lowest mechanical thresholds of the beddings tested.[166] The type of bedding used should be carefully considered if animals will be tested using behavioral somatosensory assays.[166] Another study found male C57BL/6J and ICR mice prefer cloth to paper or wood chips as bedding material and prefer the environment to be the same color as their fur, which may be related to animal welfare.[126] Thus, the type of bedding used can have major effects on study results and should be carefully selected to minimize study interference.

Diet

Apart from cold stress, diet may be one of the most important external factors affecting research mice. Research animal diets are manufactured with ingredients that are not usually found in the species' natural diets. Wild mice fed a standard research rodent diet showed a significant change in the gut microbiota within 2 wk, with a reduction in microbiota diversity over time, whereas male C57BL/6 mice 10 to 12 wk of age fed a wild mice diet (a mix of commercial wild bird seed and freeze-dried mealworms) showed an increase in gut microbiota diversity.[198] Alpha and Beta diversity in the research mice was significantly lower than that of wild mice, with *Helicobacter* species as the predominant enriched taxa in feces from wild mice and members of the family Muribaculaceae predominant in research mice.[23,198] In addition, the Muribaculaceae species found in research rodents had higher potential to be inflammatory or invasive as compared with the Muribaculaceae species found in wild mice.[23] The composition of the intestinal microbiota can also affect intestinal permeability, production of inflammatory mediators, and responses of immune cells in extraintestinal sites.[57,241] One study showed that C57BL/6NTac, C57BL/6J, and C57BL/6NCrl mice reconstituted with natural microbiota had less inflammation and greater survival after infection with influenza virus and improved resistance against mutagen/inflammation-induced colorectal tumorigenesis.[204] Gut microbiome variation can also occur within the same mouse strain depending on the source of the mice because animal husbandry may differ between institutions.[57,137,202,225]

Food colorants are sometimes used by commercial rodent diet vendors to distinguish special diets from each other. A recent study found that the common food colorant Red 40 can trigger the development of inflammatory bowel disease-like colitis in transgenic mice with increased IL-23 expression.[90] Increased IL-23 expression led to generation of activated $CD4^+$ T cells that expressed interferon-γ and could induce colitis when transferred to *Rag1*$^{-/-}$ mice exposed to Red 40. The induction of colitis was dependent on commensal microbiota that promoted the azo reduction of Red 40 and generation of a metabolite, 1-amino-2-naphthol-6-sulphonate sodium salt.[90] Researchers should be aware of possible side effects of food colorants when conducting studies. A commercially available amino acid–defined rodent diet with high folate was found to be deficient in vitamin K, which resulted in anemia, gastric hemorrhage, and mortality in male INS-GAS mice on an FVB/N background that had been fed the special diet and treated with antibiotics to prevent *Helicobacter pylori*–induced gastric carcinogenesis.[195] The study showed that antibiotic treatment reduced the abundance of menaquinone producers in the orders Bacteroidales and Verrucomicrobiales, resulting in reduced enteric production of vitamin K.[195] This study highlights the role of diet and the microbiome in maintaining vitamin K homeostasis.

The amount and type of fat in rodent diets is also important. The American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet recommends an n-6:n-3 ratio of 1 to 6:1 but no more than 7:1.[199] However, because they use vegetable oils as main source of fat, most commercial rodent diets have a higher n-6:n-3 ratio, which is known to promote inflammation and allergies.[36,117] The common practice of feeding mice ad libitum usually results in overeating and obesity, further promoting a proinflammatory state, chronic diseases, and a shorter life span.[10,89,94,219] A high n-6:n-3 ratio in rodent diets may contribute to the etiology of ulcerative dermatitis in female C57BL/6J mice.[82] A recent study compared the effects of different sources of dietary fatty acids (canola, fish, and soybean oils) on gene expression in liver of pigs and found that up to 148 differentially expressed genes were associated with metabolism, metabolic and neurodegenerative disease pathways, inflammatory processes, and immune response networks.[61] Similar studies are required in mice. Mice are omnivorous, which means that they eat both plant and animal matter. The main components of the diet of wild *Mus musculus* were seeds of foxtail grass (*Setaria* spp.) (20%), lepidopterous larvae (14%), corn (13%), miscellaneous vegetation (8%), wheat seeds (7%), and smaller amounts of various weed seeds and other insects.[243] Studies in other parts of the world have found similar diets for feral *Mus musculus* diet that reflect available foods in each season and habitat and are composed mainly of grass seeds, cereal grains, nonseed plant tissue (green leaf and stem tissue was found in large quantities in mouse stomachs only when seeds and grains were scarce), and invertebrate tissue consisting mainly of insects, particularly lepidopteran larvae (caterpillars).[21,143,216] *Setaria* spp. are annual grass plants whose seeds have a high nutritional value and health promoting properties in mice.[260] The seeds have a high linoleic acid (n-6) content but their total fat content is low, around 4%, with crude protein close to 11%.[149,260] Edible Lepidoptera generally have a high fat content (between 13% and 33%), high levels of n-3 alfa-linolenic acid (up to 45% of the total fatty acids), and lower levels of n-6 (4% to 20%) and a protein content between 13% and 74%, depending on species and stage of development.[207] Therefore, the n-6:n-3 fatty acid ratio in the natural diet of feral

mice is most likely lower than the ratio found in commercial rodent diets. In addition, the fatty acid profile of insects is very different from the fatty acid profile of vegetable oils, although the significance and/or role of these less studied insect fatty acids in mice is unknown.

Another common ingredient in commercial rodent diets is soybean meal. Soy, which provides the main source of protein in commercial rodent diets, contains phytoestrogens that are known to increase the relative uterus size in females and produce smaller embryo yields as compared with mice fed a phytoestrogen-poor diet.[196] Several studies show that dietary phytoestrogens modulate cell-mediated immunity and type I inflammatory responses, may be a factor in disease resistance and susceptibility, and may affect behavior.[40,86,87,146,252,258] Soy-free diets have been suggested as an alternative for studies in which phytoestrogens would be a confounding factor.[154]

In summary, commercial rodent diets change the natural gastrointestinal microbiome, thereby affecting multiple metabolic processes, and may be responsible for some spontaneous diseases or phenotypes, contributing to study irreproducibility. Further studies are required to evaluate the possible contributions of commercial rodent diets to allergic, autoimmune, or neoplastic diseases commonly seen in research mice, and their possible effects on research by modulating the immune system. The diet and gut microbiome composition should be reported in materials and methods sections because they can have a major effect in study results.

Water

Drinking water is also an important extrinsic factor in animal studies. Often unreported, or not reported in detail, drinking water can be a significant source of variability in animal research depending on geographical location of the animal facility and the treatment method used to inhibit bacterial growth.[15] Municipal tap water, hyperchlorinated, acidified, autoclaved, UV sterilized, reverse osmosis, etc., are all used as drinking water for animals used in research, potentially affecting the gut microbiome and altering the animal's phenotype, in particular, for studies involving motor behavior, neuropathology, and diabetes.[15,122,135,218,242,250] The pH of drinking water affected the acquisition of microflora and the overall composition of the gut microbiome in NOD mice, with those drinking acidified water showing more inflammatory T cells and relatively greater expression of inflammatory cytokines and transcription factors in the intestinal mucosa as compared with mice receiving autoclaved neutral water.[218,250] Moreover, the effects of acidified drinking water on the behavior and gut microbiota of 129S6/SvEv mice depends on the acid used for acidification.[242] Mice that received acidified drinking water from weaning did not develop the impairment in pole climbing ability shown in a mouse model of infantile Batten disease.[135] Histopathologic analysis of the brains showed that acidified drinking water reduced the amount of lysosomal storage material and astrocytosis in the striatum and somatosensory barrelfield cortex and attenuated microglial activation in the thalamus; marked changes in gut microbiota indicated a potential contribution of gut bacteria to the therapeutic effects of acidified water.[135] Investigators should report in detail the source and treatment provided to the drinking water to facilitate reproducibility of animal studies.

Ear Tags

Metal ear tags are commonly used to individually identify mice and other research species. These ear tags are composed of a nickel-copper alloy called "monel metal."In addition to nickel and copper, they also contain smaller amounts of iron and manganese.[129] Mice, rats, and guinea pigs commonly develop auricular chondritis in association with the use of metal ear tags.[129-131,161] Studies in C57BL/6 mice suggest that the auricular chondritis is a result of metal ions released from the ear tags;[129] tagged ears showed a visible increase in thickness of the pinnae and increased concentrations of copper and iron as compared with untagged ears.[129] Histologically, severe chondritis with extensive granulomatous inflammation, newly formed cartilage nodules, and osseous metaplasia with cellular infiltrates (CD4 T lymphocytes, macrophages, neutrophils, and mast cells) and expression of Th1 cytokines (IFN-γ, TNF-α, and IL-2) in the tagged ear.[129] Subsequent cellular interactions (CD4 T cells, macrophages, fibroblasts, and mast cells, mediated by cytokines such as TNF-α and IFN-γ) caused an autoimmune response that may lead to the progression of auricular chondritis as an autoimmune disease.[129,161] In humans, nickel is the most common allergen in patients with allergic contact dermatitis and atopic dermatitis.[227] Recently, ear tags made of aluminum and stainless steel were introduced into the market with the manufacturer claim that they do not cause allergic reactions in rodents. These materials should be used with caution because aluminum is known to cause contact dermatitis in humans, and stainless steel contains nickel, which appears to be the main cause of allergic reactions observed in human patients with stainless steel implants.[124,158,178] Such effects can clearly interfere with both research and animal well-being, and alternative means of identification should be considered.

Disinfectants

Quaternary ammonium compounds (QACs) are commonly used as surface disinfectants in animal facilities. Generally considered safe and used in many household products, the safety of QACs has become debatable because of a report that described reduced reproductive performance in mice coincident with the introduction of a disinfectant containing both alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride.[105] That report described the serendipitous discovery that mice exposed to QACs (ADBAC, n-alkyl dimethyl benzyl ammonium chloride, and DDAC, dodecyl dimethyl ammonium chloride) in food showed reduced fertility.[105] Further studies showed these 2 quaternary ammonium compounds were present in caging for several months after the cessation of disinfectant use.[163] The investigators reported that exposure of C57BL/6J and CD-1 mouse breeding pairs to the disinfectant for 6 mo was associated with reduced fertility and fecundity: male mice exhibited declines in both sperm concentration and motility; female mice spent significantly less time in estrus and showed longer times until the first litter, longer interpregnancy intervals, fewer pups per litter, fewer pregnancies, and significant morbidity in near-term dams.[162,163] These disinfectants have also been associated with changes in sterol and lipid homeostasis and neural tube defects in C57BL/6J and CD-1 mice neonatal brain.[92,100] In humans and female BALB/cJ mice, chronic exposure to QACs increases the risk of developing asthma and chronic obstructive pulmonary disease.[18,56,141,165] In addition, mixing QACs exponentially potentiates allergic responses in the lungs of female BALB/cJ mice.[141] QACs can also alter rhe immune responses of female BALB/cJ mice, male C57BL/6J mice, and other mammals.[1,140,191]

Both alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride have been shown to induce moderate but significant genotoxic effects in eukaryotic cells at concentrations that can be found in wastewaters, indicating that

their release into the environment may cause genetic damage in exposed organisms.[62] Another research group reported that the use of didecyl dimethyl ammonium chloride was associated with an increase in chromosomal abnormalities in a nonhuman primate colony.[47] Human exposure to QACs has increased since the start of the COVID-19 pandemic, and recent studies show the levels of QACs in human blood are significantly higher than those present before the pandemic; the detection of QACs in human breast milk led to concerns about early exposure of nursing infants through breastfeeding.[261,262] The disinfectant manufacturing industry claims these 2 QACs are safe in rats, rabbits, and humans.[97,98,152] However, an extensive literature supports the fact that QACs persist in the environment and can be toxic to humans and many other vertebrate and invertebrate species.[18,47,56,62,92,100,101,105,118,128,140,141,162,163,165,184,191,256,262] Mouse breeding facilities should consider the potential effects of these 2 specific compounds on their animal colonies and possibly their workers after prolonged exposure. Other, less toxic, commonly used disinfectants were found to affect the gut microbiota of female C57BL/6N mice and should also be carefully evaluated before use and described in publications.[214]

Environmental Enrichment

Environmental enrichment (EE) that allows species-specific behaviors is necessary for psychologic well-being and also promotes physical activity.[181,226] EE that promotes normal species-specific behavior is recognized as important for neuroscience research but that is not always the case for other areas of research.[8,20,66,76,78]

Using cages that are larger than recommended does not appear to significantly improve mouse well-being as compared with housing density.[168,244] One study found that larger cages did not improve reproductive performance in C57BL/6 mice.[244] Another found that C57BL/6J and BALB/cJ mice housed at higher than recommended density from weaning to 5 mo of age had significantly reduced growth rates, increased adrenal gland size, higher concentrations of fecal corticosterone metabolites, and increased anxiety and barbering.[176] In contrast, another study housed 5 strains of mice (129S1/SvImJ, A/J, BALB/cByJ, C57BL/6J, and DBA/2J) at the density recommended by the Guide and at densities that were approximately 2, 2.6, and 3 times greater; these mice were evaluated throughout 3- and 8-mo periods for health and well-being and found that housing density had no significant effect on the outcome measures.[113,168] Among 27 traits measured, kidney weight, adrenal weight, and heart rate decreased in mice that were housed more densely, but values remained within normal physiologic ranges.[168]

Housing density and the availability of materials that will allow the animals to perform the species-specific behavior appear to be more important to the animals than cage size. A study housed breeding trios of C57BL/6Tac mice in 2 different sizes of cages ("standard" and "large," with 82 and 124-sq-in floor space, respectively).[244] Half of the cages of each size contained 4 enrichment items (cotton square, plastic tunnel, nylon rings, and running wheel), whereas the other cages had no enrichment. Pups raised in large cages weighed less than those raised in standard cages. Male pups born in enriched cages showed more anxiety-like behavior and less exploration than did males born in nonenriched cages. Although being raised in enriched or large cages did not clearly improve pups' performance in behavioral tests, enrichment (regardless of cage size) did significantly benefit reproductive performance; pups from nonenriched cages weighed less than pups from enriched cages, and fewer survived to weaning age.[244] Another study found providing a shelter to group-housed male BALB/cJ mice increased longevity and maintained low levels of aggression, whereas adding a running wheel increased aggression as compared with the shelter alone.[222] The authors suggested shelters should be considered as routine EE for group-housed BALB/cJ males.[222] Another study found that providing either a paper shelter and rolled paper bedding or an igloo with an exercise wheel in addition to the shelter in addition to cotton nesting material shortened the interlitter interval of BALB/cAnNCrl mice and increased the number of pups weaned in 129S2/SvPasCrl mice.[245] Another study found that EE provided by placing a mouse igloo in the cage activated antitumor immunity in 6-wk-old female B6C3F1 transplanted with a tumor cell line derived from an ovarian granulosa cell tumor from the same mouse strain.[223] Another study tested the effect of 2 types of EE, nesting material and shelter, on aggressive behavior in male BALB/cAnNCRLBr mice after cage cleaning and after a 1 h of isolation.[229] Nesting material reduced aggressive behavior, whereas a shelter increased aggressive behavior as compared with control housing. Furthermore, mice with shelters gained less body weight, drank less, and showed higher corticosterone levels, while those with nesting material ate less. The authors concluded that the availability of nesting material reduces aggression in male BALB/cAnNCRLBr mice and therefore may be beneficial to their physical health and psychologic well-being.[229]

Some research suggests that rodents maintained under vivarium housing conditions are not physiologically or psychologically normal;[156,181] they are often metabolically morbid (that is, sedentary, obese, and glucose intolerant and suffer from premature death).[8,156,257] Mice housed under standard laboratory conditions are sedentary, have continuous access to food, and have no environmental stimulation.[156] Under these conditions, the mice progressively gain weight during their adult life and have elevated levels of energy regulatory hormones and factors such as glucose, insulin, triglycerides, low-density lipoprotein, cholesterol, and leptin.[156] Metabolic disruption and obesity also contribute to activation of inflammatory processes in metabolically active sites such as adipose tissue, liver, and immune cells, resulting in increased circulating levels of proinflammatory cytokines and other inflammatory markers that contribute to accelerated aging and a shorter life span.[156,257] Obesity also affects the gut microbiome.[123] An animal in this condition may be a useful good model for overweight and sedentary human subjects but may be inappropriate as a model for humans with normal weight.[156] On the other hand, exposure to EE improves immune function and decreases oxidative-inflammatory processes of immune cells, particularly affecting in older mice and extending lifespan as compared with mice housed without enrichment.[8] Concerns have been raised that EE could introduce variability into study outcomes, but using a metabolically morbid animal could also confound some studies, particularly those involving neurologic, immunologic, infectious disease, and tumor research.[123,156,157,228,257]

Choosing the most beneficial EE requires careful consideration. EE should promote normal species-specific behavior and physical activity, be selected with consideration of the mouse strain and sex, and be reported in detail in research publications to help increase study reproducibility.[226]

Group Housing

Mice are social species and as such the Guide recommends social housing for mice.[113] Social isolation is known to be deleterious to the health of social species.[29,37,169,183] A change from group housing to single housing induces stress and mild

immunosuppression in adult CD1 male mice; therefore, if mice need to be separated for experimental reasons, this factors should be considered.[183] Social isolation promotes weight gain, increases food intake, increases adiposity, impairs glycemic control, reduces insulin signaling, exacerbates systemic and adipose inflammatory responses, and induces a molecular signature characterized by downregulation of several genes involved in energy balance, stress response, and neural inflammation in the hypothalamus in young C57BL/6 and BALB/c male mice.[194] One study placed a perforated transparent wall that allowed visual, acoustic, and olfactory contact between mice in a cage to separate paired female C57BL/6JRj mice; separating the pairs increased nesting and burrowing behavior as compared with singly housed mice but locomotor activity decreased; this was considered by the authors to be a short term stress response.[93] Male Brs3tm2Rei/6J that were singly housed at 23 °C had lower body temperatures and unchanged metabolic rates as compared with group-housed mice.[217] In contrast, singly housed female Brs3tm2Rei/6J mice increased their metabolic rates and maintained a body temperature similar to that of group-housed mice.[217] Another study found that single housing negatively affected trabecular and cortical bone in adult male, but not female, C57BL/6J mice.[169] Another study found that the duration of social deprivation affects free-choice morphine consumption in C57BL/6J mice.[37] Single housing after weaning increases cellular apoptosis, myelination defects, synaptic protein loss, IL-1β expression, activation of the NFκB pathway, and microglial activation in the hippocampus and medial prefrontal cortex in male CD1 mice as compared with group-housed mice.[29] Male B10.BR mice, a model of spontaneous ankylosing enthesopathy, do not develop the condition when singly housed.[240] Another study found that progesterone receptor-expressing neurons in the ventromedial hypothalamus are critical for causing territorial aggression in male 129/SvEvTac mice.[251] These neurons can drive aggressive displays in solitary males independent of pheromonal input, gonadal hormones, opponents, or social context. However, these neurons cannot elicit aggression in socially housed male mice that intrude in another male's territory unless their pheromone sensing is disabled.[251]

In contrast to the benefits of social housing, group-housed mice, particularly males, often fight, in some cases causing in extensive damage. Fighting is more common in some mouse strains than others, but it can seriously affect research in which it occurs. In addition to fighting, which alone can cause stress and immune alterations, the resulting skin trauma causes numerous physiologic changes.[65,164] Male mice are territorial by nature, and they do not usually share a nest box.[39] However, under crowded conditions, mice form a social order with territories occupied by breeding pairs and groups of subordinate males.[39] Group-housed low-ranking ddY and C57BL/6 male mice have low sperm count and motility or are sterile.[136,148,236] Chronic social defeat stress induces behavioral changes, gonad atrophy, and reduced semen quality in male C57BL/6 mice.[148] Obviously, such mice are poor breeders. Even when fighting does not occur and a hierarchical order is established, dominant and subordinate male mice show profound differences in the immune response.[11,12] One study found that dominant male CD-1 mice shift toward an adaptive compared with innate immunity phenotype, whereas subordinate males have higher concentrations of plasma corticosterone than do dominant males.[145] Dominant mice show a relatively higher expression of specific genes involved in urine production and catabolic processes, whereas subordinate mice show relatively higher expression of genes promoting biosynthetic processes, wound healing, and proinflammatory responses.[145] In addition, subordinate male CD-1 mice show relatively higher expression of genes facilitating oxidative phosphorylation and DNA repair; corticosterone was negatively associated with genes involved in lymphocyte proliferation and activation.[145] Chronic social defeat in male C57BL/6 mice induces behavioral changes and reduced richness and diversity of the gut microbiota, with distinct shifts at the level of operational taxonomic units across phyla.[19] Defeated mice also exhibit sustained alterations in dendritic cell activation, and transient elevations in numbers of IL-10$^+$ T regulatory cells.[19] Another study reported that dominant and subordinate male BALB/c mice show increased serum corticosterone and proinflammatory cytokines during social interactions, but their response to pain is affected with social status.[3] Repeated social defeat enhances neuroinflammatory responses and causes prolonged sickness after innate immune challenge in adult male C57BL/6 mice.[249] Subordinate 6-wk-old male OF1 mice showed high levels of interleukin-6 and interleukin-1β expression in several cerebral structures and low expression of tumor necrosis factor-α in the prefrontal cortex.[121] Social stress in 6- to 8-wk-old male C57BL/6 mice, in the absence of any immune challenge, activates dendritic cells, increases cytokine secretion in response to Toll-specific stimuli, and renders dendritic cells glucocorticoid resistant.[192] Social disruption results in the generation of immunogenic dendritic cells that can enhance adaptive immunity to influenza A/PR/8/34 infection in 6- to 8-wk-old male C57BL/6 mice.[193] Subordinate adult male C57BL/6 mice have decreased basal neutrophil oxidative burst, NK cell activity, and resistance to B16F10 tumor growth.[190,208] Group-housed female mice also establish social hierarchies, using fighting, chasing, and mounting behaviors to establish social relationships.[247] One study found that dominant 7-wk-old female CD-1 mice had prolonged estrus cycles as compared with subordinate females, whereas subordinate females had significantly higher levels of basal corticosterone than did dominant females.[247] Subordinate female CD-1 mice also had elevated hypothalamic expression of genes that are known to modulate social behavior moderated by the action of estrogen.[247] Housing mice in groups should be carefully evaluated and monitored based on the sex and behavioral characteristics of the strain and should be described in detail when reporting studies.

Human Handling

An external factor that has been known for many years but has received little attention by the scientific community is the effect of human handling on mouse well-being. For example, a 1990 publication suggested acclimation of animals to handling and experimental procedures will produce animals that are easier to handle and that react to the experimental stimulus rather than to the handler, thus producing higher quality and more robust data.[205] Another group suggested that routine laboratory procedures commonly performed in animals such as handling, blood collection, and orogastric gavage could be stressful.[13] Another study reported that BALB/c, C57BL/6, and CD-1 mice of both sexes that were handled by the tail, with the body weight supported on the hand or arm, showed the least voluntary interaction with the handler.[107] By contrast, voluntary interaction was longer in all tunnel-handled mice and in cupped CD-1 mice and female BALB/c mice. As compared with tunnel or cup handling, tail handling also induced more urination and defecation during handling, a higher frequency of stretched postures, and fewer entries into the open arm on an elevated plus maze, suggesting stress and anxiety. The authors suggested that using tunnels to handle mice provides

an alternative means to accustom mice to being picked up.[107] Another study found that frequent handling of male and female C57BL/6NCrl mice by the tail reduced burrowing and increased despair-like behavior (measured as immobile behavior in forced-swim test) in male mice, whereas females seemed unaffected.[212] Instead, females exposed to a low frequency of handling showed an increase in fecal corticosterone metabolites; this effect was not detected in males.[212] Another study found that the handling method can affect the phenotype in mice used to model disease.[182] Picking up mice by the tail, with a tunnel, or with open hands was associated with more severe symptoms in a mouse model of glomerulonephritis as compared with unhandled mice. Female mice handled by their tails showed more severe symptoms of glomerulonephritis symptoms than did the control group.[182] In addition, plasma corticosterone was higher in C57BL/6 and BALB/c mice in the tail-handled mice group as compared with control mice. The authors concluded that handling causes stress in mice that can alter disease phenotype in mice.[182]

Conclusions and Recommendations

The reproducibility of published results is a major issue in the scientific community. Investigators, animal care personnel, and institutional animal care and use committees should be aware of the effects of external factors on animal physiology when designing and interpreting experiments. For these reasons, publications should include detailed descriptions of the animal husbandry conditions so that other research groups can replicate the conditions, leading overall to improved study reproducibility. Table 2 lists what we recommend as the minimum information for Materials and Methods sections when describing mouse husbandry. The list includes and complements recommendations of the ARRIVE Guidelines,[189] the *Guide for the Care and Use of Laboratory Animals*,[113] the 2022 NIH "Rigor and Reproducibility of Animal Studies: Extrinsic Factors Workshop,"[173] and a 2020 article titled "Micro- and Macroenvironmental Conditions and Stability of Terrestrial Models."[144] In addition, some extrinsic factors merit further study and may require modifications to current housing guidelines for mice and, possibly, other research animal species.

Table 2. Recommended minimum information in Materials and Methods section when describing the husbandry of experimental mice

Animal facility geographic location (coordinates and altitude)
Animal room temperature and relative humidity
Animal room air changes per hour
Animal room lighting (type, wavelength, illumination, and cycle)
Contact bedding (type, amount provided, catalog number, and manufacturer)
Nesting material (type, amount provided, catalog number, and manufacturer)
Caging (type, intracage air changes, catalog/model number, and manufacturer)
Cage-change frequency
Social environment (housing density/group size and composition)
Diet (type, catalog number, lot number, manufacturer)
Drinking water (source and treatment method)
Animal identification method (that is, metal ear tag, tattoo, microchip, etc.)
Environmental enrichment (type, catalog number, manufacturer)
Sanitation of and disinfectants used in the animal facility (product name, manufacturer)
Method used to handle mice and sex of the experimenter
Microbiologic status of the animals (list of tested and excluded potential rodent pathogens and information on the animals' gut microbiome composition).

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