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***Extrinsic Factors Affecting Animals and Research Reproducibility***

# **Cheleuitte-Nieves and Lipman. Improving Replicability, Reproducibility, and Reliability in Preclinical Research: A Shared Responsibility, pp. 113-119**

Domain 3: Research; T3: Design and conduct research; K11: Scientific writing and K12: Replacement, Reduction and Refinement

SUMMARY:  A myriad of scientific reports and reviews have indicated that the quality, reproducibility, and translatability of preclinical research translates to the unfortunate failure of drugs during human trials. With regard to publishing valid scientific data, authors recognize some reasons for the lack of reproducibility: inadequate study design and planning, deficiencies in animal research reporting, publication bias (reporting of positive results) and lack of use of the 3Rs (replacement, reduction, and refinement).  The authors recognize that reproducibility (different group, different experimental set up) is different from repeatability (same team, same experimental set up) and replicability (different team, same experimental set up). If results are to be reproducible and reliable, then both the investigative team and members of the animal care use program should be involved in a properly designed study. Intrinsic factors that might impact preclinical study findings include genus, species, sex, strain/substrain, knockouts and transgenic animals, reproductive status, age/weight, animal source, and health status. All of these should be reported in the methods, along with properly accepted nomenclature.  In addition, there are a number of extrinsic factors that can impact experimental outcomes. These include variables in the macroenvironment (such as temperature, humidity, lighting, ventilation, noise, vibration, cage components and density, and type of housing); diet and water source/quality/conditioning.  Most of these factors are also reported in the methods of a scientific report. In light of the issues discussed, Norecopa prepared a comprehensive checklist for experimental planning (the PREPARE guidelines) that are readily available to researchers.  In addition, NC3Rs has generated a set of guidelines for proper reporting recommendations (the ARRIVE Guidelines). The NIH recently had a workshop to identify areas where rigor, reproducibility, robustness, and transparency could be improved.  Several principles were developed to help guide researchers to improve research planning and execution.

QUESTION

1.  Which of the following is NOT a principle that can be used to include rigor, reproducibility, robustness, and transparency with research animal models?

a.   Rigorous statistical analysis

b.  Transparency in reporting

c.   Data and material sharing

d.   Providing data to a centralized database for public use

e.   Establishing best practice guidance for image-based data

ANSWER

1.  d

# **Lee et al.** [**Micro- and Macroenvironmental Conditions and Stability of Terrestrial Models**](https://academic.oup.com/ilarjournal/article/60/2/120/5929841)**, pp.120-140**

SUMMARY:Environmental variables have both predictable and unpredictable effects on research The NIH Plans on Reproducibility, ILARS work on Reproducibility Issues in Research with Animals and Animal Models, as well as the UK's NC3R's all speak to environmental factors as being important in experimental design. The NC3R ARRIVE guidelines go so far as to spell out the environmental details that should be included in study methods descriptions**(**when (e.g., time of day), where (e.g., home cage or laboratory), housing type, bedding material, number of cage companions, husbandry conditions, light/dark cycle, temperature, and availability of feed ) The American College of Laboratory Animal Medicine’s (ACLAM) Position Statement on Reproducibility is that “no detail of [animal care] should be deemed too small for sharing.” Proper environmental controls have the potential to contribute to 2 of the 3Rs: (1) enhanced reproducibility reduces animals used in experimentation to the lowest number of animals needed, and (2) refinement of housing conditions to minimize stress, prevent distress, promote and sustain normal behavior

Conditions whose values in and around the micro-environment are typically greater than the macro-environment are ambient temperature, RH, ammonia, carbon dioxide, and allergens.  Parameters that are identical between the macro- and micro-environments are barometric pressure and magnetic field effects.

The Guide for the Care and Use of Laboratory Animals (the Guide) standards and generally in the vicinity of 22°C, depending on occupant choice and species. To enable this result, fresh, outside air is usually heated or chilled to a specification programmed in the range of 10°C–16°C by the air handling system for distribution via the air supply ducts to individual rooms. At the point of delivery to each room, heat is usually supplemented via a terminal, hydronic reheat coil. These are sized to supply heated air up to 29°C–35°C, allowing for response to the thermostat when room temperature drops below the programmed set point. Humidification and dehumidification typically are managed at the central system or on a zonal basis; if not, there can be profound seasonal variation in indoor RH. A properly designed and functioning heating, ventilation, and air conditioning (HVAC) system will, ideally, fully and equally ventilate all areas of the room and respond to variations in climatic conditions outdoors or changes in the number and kind of animals and equipment indoors to maintain dry-bulb temperatures of ±1°C from individual room thermostatic set-point across the span of 18°C –29°C. An adequately functioning system will control RH within ±10% of a set-point in the range of 30%–70% year round. The ideal design prevents disturbances from physical plant maintenance by enabling routine or emergency maintenance and access to valves, filters, dampers, connections, and power to be accomplished outside of the animal housing and procedure rooms and will exclude pests so that studies are not impacted by using pesticides around animals or adding risks of infection or infestation introduced by vermin.

Temperature: The temperature supplied to the room by the HVAC is generally slightly to significantly lower than that in proximity to the animal. A number of factors influence this relationship, most importantly caging type, population density, type of contact bedding, and the presence of nesting material and/or shelters. In general, these factors add 1°C–2°C. Importantly, there are temperature gradients across all 3 dimensions of most rooms, with the macro-environmental temperature generally warmer toward the ceiling due to the relative buoyancy of hot air relative to cold and any heat emitted from ceiling light fixtures. The temperature at which energy-conserving behaviors and physiological changes are in equilibrium with the heat loss to the environment (entropy) is referred to as the thermal neutral zone (TNZ). Below the TNZ, endotherms commonly generate additional biological heat via thermogenesis. (rodents use brown fat).. In the extreme, heterothermy can result in a reduced resting metabolism along a spectrum of hibernation, torpor, or torpor-like states with reduced energy demand. Especially important to the laboratory setting, mice will exhibit these phenotypes below their TNZ and are best characterized as “facultative daily heterotherms” rather than true endotherms, that is, the thermal physiology of the mouse is dependent on the context of their environment (e.g., bedding, social huddling, environmental temperature, etc.), time of day (circadian), and not fixed at a specific body temperature. In fact, a torpor-like state is only avoided in laboratory settings due to the common ad lib feed availability. The physiological state of a laboratory animal is not only a function of temperature, RH and ventilation, housing system, and husbandry program [38], but also phenotype (e.g., hirsute or nude), body condition, physiological state (e.g., circadian rhythm), recovery from anesthesia/surgery, life stage (including age, pregnancy, and lactation status, available insulation (e.g., nesting and bedding materials), the ability to huddle to reduce collective exposed surface area, and the ability to select ambient temperature (thermotaxis).

A risk to all thermal environmental compartments is the failing of the terminal reheat system (TRS). By design, these components of the HVAC system regulating room temperature will fail open, closed, or in place with varying ramifications. If they fail closed (the Guide preference), the consequence is that fresh air is supplied to the rooms at 10°C–16°C exiting the air handling unit (AHU) unresponsive to the thermostat. Animals then are exposed to these temperatures indoors, possibly requiring animal care program intervention (e.g., relocation of the census to a warmer location) until the system is repaired. In the experience of the authors (M.J.H., V.L.) with mice in this scenario, providing there is group housing for huddling and ample nesting material, passive observation suggests that the animals behave normally and are not obviously affected. Given the importance of temperature on biological processes, it is imperative that scientists be informed of these sorts of failures so that they can account for the variability in the conduct of any experiment. For TRS that fail in place, providing it did not occur at or near an extreme of a fully opened or closed valve, the mean temperature will vary from the programmed set-point, and there may be greater variability around that mean due to the effect of the inflexible TRS. The real hazard, however, is in the TRS that fail open with 82°C water (or steam) circulated through the coil. In a room receiving 10 ACH, where in theory the full volume of the air in the room is exchanged every 6 minutes, the room temperature rapidly escalates to 40°C–50°C with the ensuing specter of compromised experiments, animal deaths, and impaired breeding colony production. The importance of this is not only in proper design, but in deploying environmental monitoring systems that detect failures and emergency conditions and alarm and immediately notify responders.

Air  Quality: Air quality standards for the macro-environment are dictated by human exposure limits set by regulatory agencies with little guidance specific to the laboratory animal community. The Guide for the Care and Use of Laboratory Animals only recommends that air pollutants are minimized but does not give more specific guidance.

Depending on whether the cage is open, fully enclosed with a FT, or individually ventilated, there can be gradations in differences of air quality between that in the cage in proximity to the animals and the macro-environment. For example, a differentiating characteristic of individually ventilated caging (IVC) and gnotobiology isolators is that the supply air is cleaner than that of the room as it is highly pure, usually HEPA-filtered, and free of dust, microorganisms, and particles suspended in the air. In general and with respect to rodents, air quality is better in suspended wire cages, uncovered shoebox cages, and IVC than in static (unventilated; sometimes called isolator) FT cages.

The micro-environment of a static FT cage is more likely to contribute to research variability than the macro-environment because of the relative accumulation of contaminants contained by the FT lid and the high variability in air quality reported. Although micro-environmental conditions are affected by the room air quality, intracage levels of gases and RH can be much higher in static relative to ventilated cages. The primary potential concerns for air quality in a FT cage are ammonia, carbon dioxide, and particulates as these are products of the animals and materials within the cage.

ACGIH = American Conference of Government Industrial Hygienists, a private, nonprofit, nongovernmental, scientific professional organization

NIOSH = National Institute of Occupational Safety and Health

OSHA = Occupational Safety and Health Administration

The drafts and cooling effects created by intracage ventilation add an additional potential variable beyond the effects of ventilation on gaseous air quality. IVC have different designs for air input and exhaust, hourly ACH, and air distribution patterns depending on manufacturer. In particular, the design and air velocity from the input valve affect the intracage air distribution, and airflow even for the same rack could change over time as the air inlets and outlets become occluded with debris. These differences in cage ventilation can lead to variations in air quality and temperature experienced by animals because intracage ventilation creates drafts and affects humidity and the accumulation of gaseous products especially relative to static cages.

Ammonia is the most common and abundant gaseous pollutant associated with rodent cages for which there is the potential for high levels with extensively documented adverse effects. Control of ammonia is particularly challenging given that many rodents, even those that are specific pathogen-free, may not be well defined regarding their inherent bacterial flora, and they may be colonized with urease-producing bacteria capable of catalyzing the conversion. Factors that affect ammonia levels beyond inherent animal characteristics include RH, caging types, and bedding. Male mice excrete approximately twice as much urine as females, and cages with male mice will be associated with higher ammonia values than females. Although the studies comparing caging type are limited, cage type has been shown to have an effect and could play a role both by containing ammonia and increasing RH levels. Urine output also increases with age and also possibly by strain. Bedding materials vary in their ability to control ammonia levels, with corncob having relatively high absorbency and demonstrated suppressive effects, followed by paper and then wood-based beddings.

Minimizing dust is a general goal within cages.

OSHA recognizes portable infrared spectrophotometers or a chip measurement system for gas level detection and includes the specific models and manufacturers commonly used for these measurements in the OSHA Occupation Chemical Database description for each chemical. OSHA recommends a particle counting instrument for airborne particulates, most commonly an aerosol photometer, also known as a nephelometer, light scattering meter, or aerosol monitor. This device relies on dust scattering light or infrared radiation to quantify particle levels.

RH extremes of <30% or >70% RH can notably influence food and water consumption, activity, postnatal development, transmission of infectious agents, ocular physiology, and the transcutaneous absorption of drugs. RH can also affect nociception and the development and progression of skin conditions, most notably ringtail in rodents, a condition ranging from focal tail tip necrosis to an extensive contortive deformity of the tail, usually, but not always, associated with low extremes of RH (i.e., dew point) in combination with the ambient temperature, thus affecting evaporative heat loss that in turn influences temperature perception.

Mice appear averse to direct airflow with IVC systems set at 60 and 100 AHC. Rats not only prefer air exchange rates of 50 ACH relative to 80 ACH and higher, but also 80 ACH and above have significant impacts on heart rate and blood pressure. Aversion and cold stress in mice seen at the higher ACH can be mitigated with protective materials such as shelters and nesting materials, although the ability for these materials to reduce potential impacts of IVC on behavioral phenotypes is unknown.

Increasing RH, particularly that above 60%, directly and significantly promotes the production of ammonia, enables the hatching of food pests, and results in condensate that fosters mold growth on feed and within the ducts of the HVAC system . The Guide does have a general standard for the macro-environment of terrestrial species of 30%–70% humidity, but higher levels might be found within static FT cages. Even with RH levels of 44%–50%, static FT cage RH levels are routinely 70%–74%. In general, ammonia production is inhibited by RH < 50%, and IVC offer the advantage of tying micro-environment RH more closely with the room.

The primary concern for ammonia as a research variable is its effects on the respiratory system.

The studies specific to rodents show wide variability in ammonia levels within routine housing conditions. With respect to the typical cage, the ammonia concentration is 0 ppm immediately after cage change, begins to increase exponentially sometime thereafter (as influenced by cage type and environmental factors), often then plateaus, and then drops back to zero subsequent to the next cage service. Among any group of cages, there will be great variation in the rate of increase and peak measurement of ammonia at any point in time. Individual cage ventilation is the most obvious method to decrease ammonia levels. Ventilated cages can be easily kept below a peak of 25 ppm even at longer cage change intervals, depending on air change frequency and as mitigated by low RH. However, more surprising is the large range in ammonia levels in static cages from <25 ppm to >400 ppm by day 7, with 1 report even up to 700 ppm ammonia.

Ammonia levels can increase the severity of experimental respiratory infections in a dose-dependent fashion from 25 to 250 ppm in rats with Mycoplasma pulmonis, and ammonia levels of 500 ppm exacerbate Pasteurella multocida infections in mice. Fewer studies have evaluated the pulmonary effects of exposure to ammonia, but none have been found with normal exposure levels.

Mouse intracage carbon dioxide levels range from 1000 to 6000 ppm in static FT cages, 900 to 2000 ppm in open cages, and <5000 ppm in IVC. These values are significantly greater than the macro-environmental carbon dioxide level of approximately 400 ppm. In contrast to ammonia, CO2 levels measured in the macro-environment are generally below the threshold for human exposure, and no research has shown experimental effects on rodents exposed to naturally occurring levels.

Bedding: Laboratory animal beddings vary in material and shape, creating the potential to add variability to animal models, with a potential for a range of effects on reproduction, behavior, physiology, dietary studies, the microbiome, and exposure to undesirable microorganisms. These effects could be particularly profound for species that ingest the bedding, such as rats and mice. No specific type of bedding is ideal for all species under all management or experimental conditions, but optimally contact bedding should be absorbent, free of toxic or aromatic chemicals, safe, nonabrasive, disposable, comfortable, nonstaining, inexpensive, and readily available, but not readily eaten. Corncob-, wood-, and cellulose-based products are the most commonly used in current facilities for rodents.

Mice prefer softer materials and those that allow burrowing and nesting, such as paper-based beddings, and both rats and mice prefer larger particles and fibrous materials relative to small particles.

Corncob, virgin cellulose, and eucalyptus pulp, inhibit ammonia production. Softwoods such as pine have been used historically and are still sometimes used following heat treatment that minimizes its impact on liver function. Pine and cedar have long been recognized as affecting drug metabolism in rodents by inducing cytochrome P450 enzymes with effects that can last for weeks, even after removing the animal from the bedding. Softwood beddings have also been shown to induce rat liver endocytosis and possibly affect other liver enzymes as well. These effects have not been found in rabbits exposed to softwood bedding, although the rabbits did not have direct contact, and bedding treatment was not described in this study. Contact beddings made from heat-treated hardwoods such as maple, birch, beech, aspen, or poplar may have residual vitamin C, which can confound studies of scurvy using guinea pigs. Bedding materials are a source of microorganisms and are often sterilized to reduce the microbial burden by either irradiation or autoclaving. Multiple types of unsterilized bedding have been found as a source of fungal spores, bacteria, and nonpathogenic nematodes. These agents may be ingested or aerosolized, presenting a risk of mucosal contact or inhalation, depending on the bedding’s characteristics such as dustiness, and can be influenced by the caging system utilized. Although even the same type of bedding can vary widely in bacterial content and no beddings are sterile after normal manufacturing processes, paper beddings are reported to have lower bacterial content and endotoxin levels compared with corncob and wood. Autoclaving not only reduces the microorganism loads to undetectable levels but also may be associated with ammonia suppression within the cage. Although extensive analysis of the bacterial flora associated with specific bedding types is limited and likely highly variable depending on material and vendor processing, the primary bacterial flora identified in corncob bedding is considered normal flora for immunocompetent mice. The risks of unsterilized bedding should still be considered given that it has been associated with fungal rhinitis in rats, and feed and bedding in shipping containers have been implicated as a source of rotavirus infection in mice. The presence of endotoxins and bacteria has been suggested as a potential confounder for respiratory-related research, and endotoxins are not as sensitive to autoclaving as bacteria.

Bedding can affect the microbiome and immune system of rodents. Bedding type has influenced dietary studies in mice and the outcome of periodontal studies and disease in rats, and it has had variable effects on rat body weight.

In rats, aspen shavings have been associated with higher rates of sneezing irrespective of ammonia levels relative to paper bedding, suggesting that differences in dust levels could be relevant. Particulates from cotton enrichment can be a source of conjunctivitis and blepharitis in nude mice.

### Barometric Pressure: Biological effects may be altered in animals as a consequence of changes in barometric pressure associated with local weather patterns, in transportation (particularly by air freight), and when relocated between low- and high-altitude sites.

### Electrical and Magnetic Fields as well as transportation  are also  mentioned in this article as having possible impacts.

This article also mentions ENVIRONMENTAL VARIABLES AND JOHAIR WINDOW KNOWNS AND UNKNOWNS ( basically new things come to light that we find are variables and then there are still things we don't know)

QUESTIONS

1. What agency published the ARRIVE Guidelines?

2. Define the following: TNZ, Heterothermy, ACGIH

3. T/F: The Guide prefers that the TRS of the HVAC fail in the open position

4. T/F: An infrared spectrophotometer can be used to measure dust

5. Which types of bedding inhibit ammonia production?

6.  Conditions whose values in and around the micro-environment are typically greater than the macro-environment are ?

ANSWERS

1. NC3R

2.  TNZ - The temperature at which energy-conserving behaviors and physiological changes are in equilibrium with the heat loss to the environment (entropy)

 Heterothermy - reduced resting metabolism along a spectrum of hibernation, torpor, or torpor-like states with reduced energy demand

 ACGIH - American Conference of Government Industrial Hygienists, a private, nonprofit, nongovernmental, scientific professional organization

3. False - prefers closed

4. True

5.  Corncob, virgin cellulose, and eucalyptus pulp

6.  RH, ammonia, carbon dioxide, and allergens

# **Sanders and Farmer.** [**Aquatic Models: Water Quality and Stability and Other Environmental Factors**](https://academic.oup.com/ilarjournal/article/60/2/141/5929830)**, pp. 141-149**

Domain 4:  Animal Care

Primary Species:  Zebrafish (*Danio rerio*)

SUMMARY: We have seen an increase in researching using aquatic animal models, including Zebrafish (*Danio rerio*) which have become a primary laboratory animal species. While the 8th Edition of The Guide for Care and Use of Laboratory Animals (2011) has a section dedicated to aquatic species more published research and guidelines addressing aquatic species is needed. The authors stress the importance of water quality on research outcomes as well as animal welfare. They also emphasize the importance of including husbandry details and water quality parameters when publishing aquatic animal model research. The article mainly refers to zebrafish facilities.

Water Quality

* Source Water – Common methods of water purification include reverse osmosis and/or deionization.

o    Reverse Osmosis – forces water under pressure through a semi-permeable membrane with small pores against a concentration gradient to remove impurities

o    Deionization – exchanges positive hydrogen and negative hydroxyl molecules for positive and negative dissolved minerals and solids in the source water

o    Purified water then needs to be conditioned by adding salt ions and buffers

* Chlorine and Chloramine - have been shown to be lethal to fish but are commonly found in municipal water. Catalytic granular activated carbon (GAC) is used to neutralize chloramines, remove tannins to maintain water clarity and absorption of some dissolved organic compounds and contaminants.
* pH – Most fish water is maintained at or near 7.5 pH (Zebrafish natural range is 6.6. to 8.2 pH). High or low PH levels can lead to increased cortisol, loss of normal swimming behavior, hypoactivity, convulsions, death, and even changes in gene expression.
* Nitrogenous wastes (ammonia, nitrites, nitrates) – Ammonia is the primary waste product (excreted across gills and in feces, and food decomposition). At higher temperatures and pH the toxicity of NH3 increases. Bio-filters are commonly used to convert ammonia to the less toxic nitrate (No2-). Common practice is to maintain a 10% water exchange rate to remove the less toxic Nitrate (NO3-) but is system dependent (stocking density, feeding practices, total volume, etc.)
* Temperature – Zebrafish are poikilotherms (cold-blooded). Accepted standard temp for zebrafish embryos is 28.5 C and 24-28 C for larvae and adults. Temperature can affect sec determination.
* Salinity and Conductivity – Salinity is the total concentration of all ions dissolved in water (recommended range 0.5-2.0 g/L). Conductivity is the amount of dissolved salts or ions in the water (range from 100-2000 micro Siemens). If ion levels are too high or too low, fish will expend excessive energy to manage tissue hydration necessary for normal physiological function. Conductivity can be maintained by using reef salts or high-purity NaCl.
* Hardness – the amount of divalent ions (Ca+2, Mg+2, Fe+2, Mn+2, Sr+2) present in fish water. They are essential for growth and physiological functions. Aragonite sand or crushed coral maintained in the system provides calcium carbonate. Recommend a minimum of 75 mg/L be maintained for Zebrafish systems.
* Alkalinity – refers to the amount of all bases present in water and reflects the pH-buffering capacity of the solution. Recommend maintaining at 50-150 mg/L CaCO3. Bicarbonate (HCO32-) is essential for the biofilters and the nitrifying bacteria. Systems use sodium bicarbonate plus the aragonite/crushed coral as an additional source.
* Dissolved Gases – Dissolved oxygen should be maintained at or near saturation (~ 7.8 mg/L at 28C). Aquatic hypoxia is considered a teratogen leading to malformation and changes in gene expression in embryos in addition to male dominated sex rations. CO2 – accumulation decreases pH and can have a narcotic effect on fish. Supersaturation in excess of 103% can lead to Gas Bubble Disease (GBD).
* Solid Wastes

o    Dissolved solids (< 2 microns) can affect water color and clarity. Arise from food and algae. Eliminated by water exchange and GAC.

o    Suspended solids (> 2 microns) can cause gill damage. Eliminated by water exchange or mechanical filtration. Settleable solids should be manually removed when observed.

o    Biofilm, Algae, Cyanobacteria – difficult to eliminate but can be managed using tank exchange, minimize intense lighting, water quality management, and sound feeding practices. No standard tank exchange rate has been established. Biofilms can harbor *Vibrio spp*., *Mycobacterium spp*. And parasites. Though not reported to have occurred in Zebrafish facilities, Cyanobacteria can produce neurotoxins, hepatotoxins, and dermatoxins which can have a negative effect on research but are also a potential occupational health hazard. Byrozoans may be pathogenic or parasitic to zebrafish and are impossible to remove from a system once present.

* Lighting and Illumination

o    Light Source – Currently no literature on fluorescent vs LED lighting exists for zebrafish facilities.

o    Light Intensity– ranges from 54-324 lux, needs to be bright enough for personnel to work and fish to detect food but low enough not to promote growth of algae.

o    Spectrum and wavelength – Zebrafish can perceive light beyond the range of humans. Recommend selecting a light closest to sunlight. Research in this area is very limited.

o    Photoperiod – Either 14:10 or 12:12 works, the important thing is that it is consistent and uninterrupted, especially for spawning.

* Zebrafish Housing Aquaria

o    Housing density – low numbers (<5 fish) or high numbers (>40 fish per liter) or where water volume in inadequate to prevent schooling behavior can lead to increased aggression and cortisol. Physical enrichment such as plants can decrease aggression but too much can increase aggression. Housing density can also affect sex determination.

o    Flow rate – needs to be adjusted for each live stage. It must be able to remove waste products but not to strong that it interferes with swimming.

o    Noise and vibration – Excessive vibration may negatively impact spawning, suppress appetite, swimming, and social behavior. Zebrafish hearing sensitivity range is 200-4000 Hz. More research is needed to establish guidelines.

QUESTIONS (True or False)

1.  Catatonic gradient activated carbon (GAC) is used to neutralize chloramines and remove tannins from aquatic system water.

2.   Water temperature, low dissolved oxygen, and housing density can affect sex determination in zebrafish.

3.  In order to decrease the buildup of biofilms and algae in tanks, the 8th edition of The Guide for the Care and Use of Animals recommends that tanks are exchanged and cleaned a minimum of once every 4-6 weeks.

ANSWERS

1. False

2. True

3. False

# **Hanifin et al. Relevance of Electrical Light on Circadian, Neuroendocrine, and Neurobehavioral Regulation in Laboratory Animal Facilities, pp. 150-158**

Domain 3: Research), Domain 4: Animal Care

SUMMARY:With the present review, the authors discuss the complex effects that light has on the neuroendocrine, neurobehavioral, and circadian physiology of laboratory animals and underline the importance of carefully consider light as a key extrinsic factor, especially in operations and in the design of animal facilities.

The review also emphasizes how research has provided a better understanding of phototransduction for circadian, neuroendocrine, and neurobehavioral regulation with the wish that these findings may help to improve lighting in animal facilities.

The data reported in the article summarized here mostly refer to studies performed on mice, rats, and hamsters, but may provide useful insights to animal facility managers and researchers working with different species.

The broad range of physiological responses within the organism that is evoked by light stimuli is known as phototransduction; the specific molecules that absorb light energy to initiate photobiological responses are called chromophores (or photopigments). A photopigment named melanopsin has been identified both in the retinas of rodents and humans and is found in the photoreceptive retinal ganglion cells (ipRGCs).

The authors give an overview of the neural pathways involved in circadian and neurophysiological regulation. Neural projections from the ipRGCs form the origin of the retinohypothalamic tract (RHT) which in mammals is the primary neural projection to the circadian oscillator for entraining circadian rhythms to environmental light-dark cycles. This pathway acts to convey information about external light conditions from the retina to several area of the hypothalamus, including the suprachiasmatic nuclei (SCN). In turn, the SCN transmits information about lighting and circadian time to different regions of the nervous system, including the pineal gland, where the hormone melatonin is synthesized.

If melatonin is considered to be the main neuroendocrine hormone to be affected by light, there are evidences showing that acute light exposure has an effect on the corticosterone levels (increase), similarly to the effects elicited by other major physical stressors. These findings should be considered in the animal facilities in which nocturnal light exposure may occur.

When considering light and its consequences on the animal physiology and behavior, it should be noted that different parameters should be considered in the design of animal facility rooms, as the periodicity of light, intensity, and wavelength.

In particular, the review reports interesting findings concerning the effect of light wavelength on circadian, neuroendocrine, and neurobehavioral regulation. The weakest stimulation of circadian and neuroendocrine responses occurs in the longer wavelengths above 550nm; however, a study in rodents shows that long-wavelength red light exposure (above 620nm) at night, if of high enough intensity and duration, results in the disruption of the circadian organization of neuroendocrine, metabolic and physiological parameters associated with the animal health and well-being. Ultraviolet wavelengths (300-380nm) also appear to elicit circadian and neuroendocrine responses in some mammalian species but according to some studies that used monochromatic light exposures in monkeys and rodents, the neuroendocrine, neurobehavioral, and circadian responses are maximally sensitive to blue light (between 459 and 483 nm).

The threshold sensitivity to light response vary among mammalian species and according to recent analysis performed in laboratory rodents, as little as 0.01 lux may be sufficient to elicit a biological response. More studies using animal models show high sensitivity to phase shifting by light, after exposure to extended period of darkness: it appears that maintaining absolute darkness during the animal’s dark phase is essential, as little as 0.2 lux of light has been shown to alter circadian rhythms , as well as to cause increases of oncogenesis via melatonin suppression in rats exposed to 0.25 lux.

Concerning the ocular and physiological elements that mediate the biological effects of light, there are 2 main sets of elements that should be considered: the first refers to physical/biological stimulus processing (light source physics, conscious and reflex behavior of the animal relative to the light source, transduction of light to the retina through the pupil and ocular media), while the second refers to the sensory/neural signal processing, which is initiated as photons are absorbed by retinal photopigments and neural signals are generated. Factors that influence the effectiveness of environmental photic stimulus for regulating the mammal physiology are the wavelength sensitivity of the photoreceptors, the distribution of photoreceptors, the state of photoreceptor adaptation and the ability of the central nervous system to integrate photic stimuli over space and time.

Some considerations are made on the current state of lighting for animal room facilities as well as the importance of reporting data on lighting in scientific publications.

If light measurements are provided, they are typically reported in lux, which indicates a photopic measurement based on the selective responsiveness of the human visual system and, as a consequence, it appears inappropriate to use it as a measure in mammalian study of circadian and neuroendocrine physiology. According to the authors, radiometric units are best used as they are based on unweighted power measures.

The current state of lighting for animal rooms facilities is in transition from primary fluorescent to solid state LED lighting. The authors underline the large differences in the distribution of wavelengths in these light sources, with more light being present in the blue-appearing portion of the visible spectrum (465-485 nm). It has been demonstrated that solid state lighting can have significant effects on pigmented nude rat circadian physiology, increasing nighttime melatonin signal. Recent studies have investigated the influence of daytime LED exposure on neuroendocrine, metabolic, and physiologic parameters in 3 strains of commonly used laboratory mice (however, the strains were not reported in the article) and data obtained showed that the effects are generally beneficial to the animal’s homeostatic regulation of health well-being.

QUESTIONS

1.   Please give a brief definition of circadian rhythms

2.   Concerning melatonin regulation, what happens during exposure to light during the night?

3.   True or False: Many mouse strains are melatonin deficient but the C3H and CBA strains are known to have detectable high amplitude melatonin rhythms

ANSWERS

1.   Circadian rhythms are defined as endogenous rhythms that influence nearly all physiological functions and with a periodicity of approximately 24 hours; they are synchronized to the physical environment by the central clock located in the suprachiasmatic nucleus and by peripheral clocks in various metabolic tissues (for example: liver, pancreas, intestine)

2. Night exposure acutely suppresses melatonin synthesis through the decreased activity of the pineal enzyme aralkylamine N – acetyltransferase.  The acute light-induced suppression of nocturnal melatonin synthesis was first observed in rats and has been used in numerous studies on mammals to help determine the neural and biochemical physiology of melatonin regulation.

3. True

# **Reynolds et al. Sound and Vibration as Research Variables in Terrestrial Vertebrate Models, pp. 159-174**

**Domain** 4

**SUMMARY**:  Sounds and vibrations are two forms of energy that are closely related.  Many species of animals interpret sounds/vibrations as potential threats and this can have an impact on their endocrine, metabolic, cardiovascular, immune, reproductive, gastrointestinal, and musculoskeletal systems.  Mitigating these changes will help to make the research results more reproducible.



Both sound and vibration are means by which energy is transmitted in waves that emanate from the source.  Sound amplitude is commonly measured using the decibel (dB) scale, which is a measure of sound pressure levels. The higher the amplitude of the sound wave, the higher the sound pressure level (see diagram).  The perception of sound a vibration by the animal will depend greatly on the amplitude and the frequency.

Sounds are transmitted as follows: tympanic membrane, malleus + incus + stapes, oval window, basilar membrane, and hair cells.  From there the auditory nerve conducts signals to the brain for interpretation.  The frequency at which the ears of each species can hear varies.   Mice and rats hear at much high frequencies than humans.  And the range of frequency at which animal can hear varies greatly from species to species.  Sound intensity levels (amplitude) and frequencies are certainly of concern, and the varying hearing frequencies between species may make determining a standard achievable limit for all testing scenarios more complex.

Which sound is a type of vibration, true vibration is perceived by mechanoreceptors in the skin that initiate a signal to the brain when the skin is stretched, or the receptor is compressed.  It is also important to know the resonate frequency of an object (including an animal!) which is the frequency at which the object itself begins to vibrate.  Unfortunately, the resonance frequency ranges for various species are generally unknown.

There are various sources of sound and vibration that one must be concerned with.  Some of them are intentional (speaker) while others are not (HVAC, motion sensors, doors, blowers, etc.).  It is important to account for these sources of sound and vibration, as they could have an effect on the experimental outcomes.  The variables that can be introduced can happen physiologically, but also at the cellular level.  Strategies to mitigate unwanted sound and vibration should be explored to reduce their effect on research.  For example, a common source of noise and vibration is the movement of animals between program areas. It was found that placement of a towel beneath a rodent cage being transported on a cart dampened vibration levels by more than 50%, and hand carrying a cage also helped.

**QUESTIONS**

1. Which of the three small bones in the ear articulates with the oval window to move the fluid in the inner ear?
	1. Malleus
	2. Incus
	3. Stirrup
	4. Stapes
2. The frequency at which a mouse’s hearing is the most sensitive is:
	1. 70-90 kHz
	2. 10-20 kHz
	3. 1-5 kHz
	4. 100-120 kHz
3. Vibration is perceived by \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ in the skin that initiate a signal to the brain when the skin is stretched or compressed.
	1. Hair cells
	2. Microsensors
	3. Mechanoreceptors
	4. Baroreceptors

**ANSWERS**

1. d
2. b
3. c

# **Kurtz and Feeney. The Influence of Feed and Drinking Water on Terrestrial Animal Research and Study Replicability, pp. 175-196**

Domain 1

SUMMARY: The research community has long recognized the need for high-quality animal feeds that can optimize growth, maintenance, and reproduction in most species.  Over time we have gained a much better understanding of the role that individual nutrients play in physiological responses and in some studies diet is often considered as an independent variable in experimental design.  More recently the importance of drinking water as a potential source of experimental variability has come under greater consideration.  As the issue of reproducibility and replicability has come under greater scrutiny the importance of diet and water as potential sources of inherent variability must be considered.

***The goal of this review is to provide background on contemporary issues regarding how diet and drinking water might serve as a source of extrinsic variability that can impact animal health, study design, and experimental outcomes and provide suggestions on how to mitigate these effects.***

Food is comprised of protein, carbohydrate and fat which are macronutrients utilized for energy; and vitamins and minerals which are micronutrients that serve as catalysts and cofactors for cellular processes.  The NRC (National Research Council) publications provide information on quality assurance, contaminants and toxicants, bioavailability, and palatability for commonly used laboratory species.

Diet formulations include natural ingredient diets, purified diets, and chemically defined diets in order of increasing cost and decreasing variability.  **Natural ingredient diets** are cereal or grain-based diets formulated from raw agricultural materials.  They are manufactured as open (complete formulation is reported and can be reproduced by any feed manufacturer) or closed formulations (composed of a proprietary mixture of components and can be least-cost, fixed or variable formulations).  Manufacturer changes to least cost or variable formulations may not be reported to the end-user.  In the 1970’s open formula diets were developed at the NIH to standardize laboratory animal diets and reduce variability in diet formulations. Since that time 13 open formulations have been developed and published by the NIH.   In 1996 the National Toxicology Program (NTP) developed an open formula rodent diet, NPT-2000 which addressed a nephropathy which was commonly observed in F344 rats used in rodent bioassays.  **Purified diets** are formulated with separate refined or purified ingredients such as casein or soybean meal (protein) , sucrose or cornstarch  (carbohydrate) and soybean or corn oil (fat).  They are also provided as open or closed formulations.  The original American Institute of Nutrition purified diet, AIN-76 was reformulated to the AIN-76A diet taking into consideration loss of Vitamin K.  More recently the AIN-93 series of diets were formulated taking into consideration a better understanding of rodent nutritional requirements.   These diets provide a more consistent nutrient profile and may be used in imaging and to better understand the importance of individual dietary components.  **Chemically Defined diets** contain more basic constituents (specific amino acids, sugars essential fatty acids and purified vitamins and minerals and are primarily used to study dietary deficiencies immune function and intestinal homeostasis.

**Physical forms of feed**include pelleted, extruded and meal or powdered diets.   Manufacture of pelleted diets includes grinding raw components and mixing vitamins and minerals.  The diet is then compressed via steam and pressure through a metal die.  Lower temperatures are used when pelleting purified or chemically defined diets.  Extruded diets are ground finer than pelleted diets which results in greater gelatinization of starches when heated.  These diets are also heated to a higher temperature than pelleted diets.  This mixture is then extruded through a die creating the desired form.  As the diet cools air pockets left behind result in a less dense pellet.  Both diets are dried to a specific moisture content.  Pelleted and extruded diets may be ground to create the powdered diet.  Soft or moist diets are generally not adequate for extended use but are available and used to encourage caloric intake for young animals, after surgery or during periods of stress.

**Feed contaminants** includingundesirable microbes and chemicals are inherent in animal feeds formulated with agricultural plant and animal products.  In 1978 the FDA recognized the need to analyze feed for contaminants that may interfere with the study.  In 1979 the EPA published test standards and maximum contaminant levels (MCL) which were updated in 1984.   Other organizations (the Organization for Economic Co-operation and Development, the British Association for Research Quality Assurance (now the Research Quality Association) and the German Society for Laboratory Animal Science all have published guidelines for MCLs in feed for rodents, but these do not carry regulatory enforcement.  More recently the Federation of European Laboratory Animal Science Associations has convened a working group to develop standards for the nutrition and contaminant limits in rodent diets.  The use of **pesticides**primarily began in the 1930’s with the Introduction of DDT.  In 1962 with the publication of *Silent Spring* environmental damage caused by DDT was brought to light and the environmental movement was started.  The most recent FDA publication reporting pesticide residues in human and animal food was in 2016.  In this report glyphosate, the most commonly used pesticide in the US was not measured.  The authors point out that the specifications on pesticides in animal feed need to be updated.  Common **microbial feed contaminants**from the use of contaminated animal products in the formulation are bacteria from the Enterobacteriaceae family, viruses and also prions.  Most, but not all of these viable microorganisms (bacteria, fungi, and viruses) are physically removed or killed during the milling process.  Sterilization of diets is necessary to kill bacteria and viruses that may have survived milling.

**Heavy metals** are natural components of the soil but can have toxic effects depending on dose, route of exposure and species.  Undesirable heavy metals such as arsenic, cadmium, lead and mercury can be found along with essential heavy metals such as iron, cobalt, manganese, molybdenum, and zinc.  Feed manufactures will certify concentrations of certain heavy metals on request.  **Perfluoroalkyl and Polyfluoroalkyl substances (PFASs)** used in non-stick and water-resistant coatings are extremely stable compounds and are under consideration as persistent organic pollutants (POP).  Published reports in animal studies unfortunately did not measure background concentrations in either food or water, however several reported detectable levels in their controls indicating and environmental source to explore.  **Mycotoxins** are metabolites produced by several common fungi that are toxic to humans and animals.  Feed contamination may occur during pre- and post- harvest processes.  **Nitrosamines** are a group of carcinogens found in cured meats and fish.  The most likely source of nitrosamines in natural ingredient diets is fish meal.

**Diet sterilization**, driven in part by the demand for specific- pathogen free animals is commonly done by either steam autoclaving or gamma-irradiation.  Both methods can reduce feed quality.  Feed manufacturers will increase heat labile vitamins; however, the percentage loss can vary based on the autoclave and cycle used.  Additionally, autoclaving feed containing high levels of starches has been shown to result in the production of acrylamide, a neuro- and genotoxin.  Irradiation of diets generally use doses of 10-30 kilogray.  Irradiation of natural ingredient diets has been shown to increase glycosinolates and the peroxidation of lipids.  Direct sterility testing of the feed is recommended.  **Diet hardness** can affect feed intake in young rodents.  Autoclaving diets may increase pellet hardness proportional to sterilization temperature.

Rodents are generally maintained in research settings using **Ad Libitum feeding**.  Most other laboratory animal species used in research are fed a daily ration.  Ad libitum feeding has been shown to negatively impact rodent health resulting in obesity, chronic renal and cardiac disease, increased incidence of neoplasia and decreased life span.  The use of restricted or ration feeding of rodents has been recommended to increase lifespan and reduce incidence of chronic disease.  This allows more animals to be evaluated at late time points in chronic studies.  Methods of dietary restriction, however, may alter biochemistry, physiology, and the circadian rhythm.

**Drinking water** for laboratory animals is generally derived from surface water or groundwater aquifers.  Most public water in the US is treated to meet the EPA’s National Primary Drinking Water Regulations (NPDWRs), however in many rural areas water may be provided by underground wells which are not covered by EPA regulations. Microbial contamination of public treated water is rare in developed countries.  Chemical contamination of public treated water or wells may include pesticides, antibiotics, hormones, endocrine disruptors, disinfectants, halogenated organic compounds, heavy metals, and radionucleotides.  Perfluoroalkyl and polyfluoroalkyl substances are common contaminants of water and are not adequately removed by contemporary municipal water treatment.  Animal facilities should document the baseline microbial and chemical contaminants of their incoming water and decide if additional purification is warranted.  Methods of purification can include filtration using granular media, resins, and porous membranes.  Additionally, carbon or charcoal filters adsorb organic contaminants, chlorination byproducts, heavy metals, and certain pesticides.  Other filters include kinetic degradation flux filters, deionization filters and membrane filters.  Overloaded or fouled membrane filters will reduce water efflux and media, or resin filters can allow breakthrough of undesirable molecules into the filtered water.  Deionization and or reverse osmosis filtration does remove essential minerals which reduces the palatability of water.  Most but not all microorganisms are removed by many of the water filtration methods used.  Steam sterilization, UV irradiation and chemical methods (chlorination and acidification) may be performed to limit or control the growth of potentially pathogenic organisms.  An additional consideration is the method that water is delivered.  Water bottles and automated water delivery systems are the most common methods to provide water to the animals.  Water bottles and automatic water delivery systems are generally efficient and non-problematic; however occasionally obstruction of a valve line, or a leaking stopper or sipper tube can result in decreased or blocked water supply or cage flooding.  Disposable water packets or water gels are also used to provide water.  If water delivery methods are used it is important to ensure that the animals are drinking.  Additional considerations include leaching or chemicals from water bottles, pipes, and sipper tubes.  Increased chemical leaching into water can occur with demineralized and acidified water.  Additionally, trihalomethanes can develop within an automated water delivery system as a consequence of chlorine disinfection and reaction with system biofilms.

Replicability is an ongoing issue in scientific research.**Reducing potential experimental variability from animal feed and drinking water**is important towards achieving this goal.  The ARRIVE (Animal Research: Reporting of in Vivo Experiments) recommends including details on housing and husbandry including information on feed and water.  The authors propose a number of steps be considered to minimize the potential influence of diet and drinking water on animal-based research studies.

1. Nutrient requirements for all laboratory animal species should be revisited to incorporate new information since 1995.
2. A global review and update of microbial and chemical contaminants accompanied by MCL.
3. Purified diets should be considered for experimental studies especially when study endpoints may be affected by diet.
4. When using natural ingredient diets open formulations should be considered to allow for transparency in reporting.
5. Animal research facilities should consider purifying animal drinking water
6. Methods for dietary / caloric restriction of rodents should continue to be explored
7. Educational outreach should be provided as to how diet and drinking water can impact animal health and influence experimental outcomes.
8. Research institutions should consider establishing a scientific review process that critically assesses all aspects of study design including diet and drinking water
9. Researchers should report sufficient detail on diet and drinking water to enable study replication
10. Journal editors and reviewers should critically assess submitted manuscripts for appropriate detail including a complete description of diet and drinking water.

QUESTIONS

1. What is a contaminant of drinking water that is not removed by contemporary municipal water treatment?
2. What are Natural Ingredient diets and how are they different from Purified diets?
3. Why is it important to sterilize diets and how may this be accomplished?
4. What are trihalomethanes and how are they formed?
5. Why is it important to provide detail on diet and drinking water in experimental studies?

ANSWERS

1. Perfluoroalkyl and polyfluoroalkyl substances
2. **Natural ingredient diets** are cereal or grain-based diets formulated from raw agricultural materials. **Purified diets** are formulated with separate refined or purified ingredients such as casein or soybean meal (protein) , sucrose or cornstarch  (carbohydrate) and soybean or corn oil (fat). They are a more defined diet useful in imaging, toxicology and studies were diet may be a factor in the outcome of the study.
3. Microbes may survive the milling process and provide a source of infection.  Steam sterilization and Irradiation are common methods to sterilize diets.
4. Trihalomethanes are a group of chemicals that form as the result of a reaction between the chlorine used to disinfect water and the natural organic material in the water.
5. As the issue of reproducibility and replicability has come under greater scrutiny the importance of diet and water as potential sources of inherent variability must be considered.

# **Brenes-Soto et al.** [**The Role of Feed in Aquatic Laboratory Animal Nutrition and the Potential Impact on Animal Models and Study Reproducibility**](https://academic.oup.com/ilarjournal/article/60/2/197/5929831)**, pp. 197-215**

Domain 4; T1

SUMMARY: Currently, there are no agreed-on set of nutritional requirements for aquatic species used in biomedical research.  Nutrition affects all life processes.  Growing animals need nutritional considerations for weight gain and consists of water, protein, and minerals required for bone and muscle growth.  In reproduction, energy balance is one of the most important nutritional factors and in fish, decreased food availability causes gonad regression leading to decrease in spawning.  Inadequate of excess nutrients can cause disease and can affect an animal’s response to resist infection.  Senescent animals have different energy requirements as well.  Aged animals generally need highly digestible diets that contain highly bioavailable nutrients.  Zebrafish are omnivorous and exogenous feeding begins around 5-6 days postfertilization.  The limiting factor is the mouth gape (about 100 um) which limits the size of feed they can consume.  Adult zebrafish can thrive on live diet such as rotifers at 250 um and pelleted diets as large as 1 mm.  African clawed frogs are obligate carnivores as adults with very short and simple gastrointestinal tracks.  Their diets are typically high in protein, moderate in fat, and low in carbohydrates.  Pelleted diets are available containing a variety of nutrient sources.  Axolotls are also carnivores and diets commonly used include earthworms, small fish, bloodworms, *Daphnia*arthropods, and commercial pelleted diets.  Diets for these species need to be balanced to avoid metabolic bone disease, hypovitaminosis A, and corneal lipidosis.  Cephalopod use in research has increased, and AAALAC international will evaluate these “higher level invertebrates.” Most cephalopods are carnivorous either actively hunting or scavengers; they adapt well in captivity to small fish, crustaceans, and mussels.  Frequency of feeding varies from 1 to 3 times daily up to 5 times daily if the researcher is trying to achieve faster growth.  Life stage is a factor in frequency of feeding with larval and juvenile fish being fed more frequently.  Amphibian feeding rates depend on passage rate and digestibility.  Cephalopods are fed ad libitum or intermittently with frequencies as often as twice daily to as low as twice weekly.  Zebrafish are commonly fed 1-5% body weight for adults and up to 300% for juveniles per day.  African clawed frog feed amounts are generally based on the frequency of feeding and they often regurgitate if overfed.  Ration for cephalopods depend on life stage, species, food type, and water temperature.  Usually 5-8% of the body weight is fed.

Variability in nutritional content of feeds could be a leading factor in issues of reproducibility of research results.  Most diets are manufactured for the aquaculture industry which is less concerned about specific levels of nutrients.  Pelleted feeds begin to leach nutrients once placed in the water and can have fewer water-soluble vitamins.  Microencapsulation can limit leaching.  Water flow of a tank and whether the feed is a floating or sinking feed, can affect how quickly it is removed and whether the animals have a chance to consume it.  Live feed is also susceptible to removal and can also introduce pathogens to the environment or animals.  *Aretemia, Paramecium,*and rotifers can transmit *Mycobacterium marinum*and *M chelonae* to zebrafish. Raw meat and organs fed to amphibians can lead to *Chlamydophila*and *Salmonella*infections.  Crayfish can be infected with *Batrachochytrium dendrobatidis*which can lead to amphibian population declines.  In cephalopods, the protozoan parasite *Aggregata octopiana*can be transmitted via crustaceans.  In addition to biological contaminates, feed can be contaminated with toxins.  Heavy metals and organic pollutants can be present in ingredients such as fish meal and oil.  Antimetabolites can be present in plant-based ingredients leading to interference with various metabolic processes.  Phytoestrogen compounds can be present, and their effects are largely unknown.  Mycotoxins are secondary metabolites produced by fungi and can cause severe health problems such as liver cancer in rainbow trout.  Presence of antioxidants in fish feed can lead to deficiencies in antioxidant vitamins leading to liver degeneration, anemia, and splenic abnormalities.  Synthetic hormones are often added to enhance breeding colors and induce breeding and effects in zebrafish are unknown.  Several research areas are affected by diet such as digestive physiology including gut morphology and physiology, cancer research, and host microbiome.  Calories consumed has been found linked to neoplastic processes.  Nitrosamines and nitrosamides can be a natural carcinogen.  Protein and fat sources play a role in influencing intestinal microbiota with fish fed either animal-sources or plant-sources diets showing differences in predominant phylum found in the intestinal tract.  Recently the International Council for Laboratory Animal Science complies guidelines called Harmonized Animal Research Reporting Principles.  These guidelines can be further improved to require additional information on nutrition which will promote scientific rigor and experimental reproducibility.

QUESTIONS

1. When does exogenous feeding begin in zebrafish?  And what is the limiting factor in feeding juvenile zebrafish?
2. What percentage of body weight is usually fed to cephalopods?
3. Live feed such as *Aretemia, Paramecium,*and rotifers can transmit what potential pathogen to fish?
4. Presence of antioxidants in fish feed can lead to deficiencies in what?

ANSWERS

1. Around 5-6 days postfertilization, mouth gape which limits size of feed they can consume
2. Usually 5-8% body weight
3. *Mycobacterium marinum*and *M. chelonae*
4. antioxidant vitamins leading to liver degeneration, anemia, and splenic abnormalities

# **Perkins and Hankenson.** [**Nonexperimental Xenobiotics: Unintended Consequences of Intentionally Administered Substances in Terrestrial Animal Models**](https://academic.oup.com/ilarjournal/article/60/2/216/5860947)**, pp. 216-227**

Domain 3: Research

SUMMARY:This article describes a selection of concerns on the effect of xenobiotics in research animals.

Xenobiotics are chemicals or compounds which are foreign to a biologic system. The compound or its metabolites may have effects (which may be distinct from their principal mechanism of action) that alter the experimental outcome. Research animals may come to contact with xenobiotics during routine conditioning or experimental procedures. The effects of toxicity depend on dose, route, and disposition, as well as immune status and species of the animal and properties of the compound. Xenobiotics may be anti-infectives/antimicrobials, anesthetics, analgesics, other drugs, and topical treatments including local anesthetics and surgical scrubs.

Concerns of xenobiotics’ effects are:

* Toxicity. Several xenobiotics, such as antimicrobials are toxic to specific animal species or systems (hepatotoxic, nephrotoxic, ototoxic etc.) or indirectly cause toxicity through destruction of commensal bacteria, especially in the gastrointestinal system.
* Mutagenic or teratogenic action.
* Alteration of metabolic pathways. Alteration of metabolism, enhancement, or inhibition of effects of other drugs.
* Alteration of immune system function.
* Alteration of behavioral and physiological parameters. For example, mice after isoflurane anesthesia exhibit locomotor and behavior deficits. Isoflurane may also have neurodegenerative effects affecting cognitive function, spatial learning, and memory.
* Organ/tissue/topical injury. For example, use of ethyl chloride as a vapocoolant in mice is not advised as it causes tissue damage, frostbites, and distress. Also, the use of surgical scrubs and alcohol should be re-evaluated in order to avoid hypothermia and overall uncontrolled temperature. Two applications of surgical scrub and rinsing with saline (instead of alcohol) are advised.

For more specific examples, please refer to the article!

QUESTIONS

1. According to Perkins and Hankenson 2019, “xenobiotics” are:

a. Organisms not native in the geographical area examined

b. Microorganisms which don’t belong in the normal flora of a species

c. Chemicals or compounds foreign to a biological system

d. Organisms or compounds foreign to Earth

2. Which is not a way a xenobiotic substance may affect experimental results?

a. When it is used as an anesthetic

b. When it has toxic effects

c. When it alters immune system function

d. When it alters metabolic pathways

3. Which compounds may be categorized as “xenobiotics”:

a. Any compounds

b. Anesthetics and anti-infectives (only)

c. Topical and systemic anesthetics and analgesics (only)

d. Teratogenic and toxic compounds (only)

4. Use of alcohol solution as final step of surgical site preparation may result in:

a. Hyperthermia

b. Bacterial overgrowth

c. Toxicity

d. Hypothermia

ANSWERS

1. c

2. a

3. a

4. d

# **Hasenau.** [**Reproducibility and Comparative aspects of Terrestrial Housing Systems and Husbandry Procedures in Animal Research Facilities on Study Data**](https://academic.oup.com/ilarjournal/article/60/2/228/5722085)**, pp. 228-238**

Domain 4: Animal Care

SUMMARY: The article summarizes animal housing aspects. All these factors should be described in study reports, as they may affect experimental outcomes.

* + Animal housing: Consider ease of cleaning, have minimal built-ins, operational efficiency, animal welfare.

o    Conventional

o    Barrier (protect animals from pathogens)

o    Containment (contain pathogens inside)

o    Quarantine (isolated from other animals, separate equipment, and air-flow)

* + Room design: for cages (no drain, no sloped floor), for hose-down pens (drain, sloped floor, hose), or for both (compromise…). Consider desired density, species, caging system.
	+ Caging systems: using dry bedding (rodents, rabbits) or hose-down (NHP, farm animals).

o    Rodent caging

§   Solid bottom, shoe-box type with bedding and nesting material, translucent or opaque (translucent enables observation of the animals vs opaque protects from light intensity)

§   Material is plastic: polypropylene (translucent or opaque, autoclaved up to 120°C), polycarbonate (transparent, becomes brittle after much autoclaving, autoclaved up to 120°C), polysulfone (3year-lifespan, up to 134°C), polyphenylsulfone (8year-lifespan, up to 143°C), polystyrene (disposable cages)

§   Polysulfone and polyphenylsulfone have natural amber/yellow coloration which results in 35% less light penetration than polycarbonate and protects from light intensity

§   Damaged cages (polycarbonate and maybe also polysulfone) release Bisphenol A and S, which are endocrine disruptors

o    Micro-isolation: ventilated or static cages. Cages must be opened in HEPA-filtered air cabinets, designed for keeping pathogens in or out depending on needs. Ventilated caging systems may have high noise and vibration and air drafts but static caging results in rapid elevation of humidity, ammonia, and carbon dioxide which in turn results in need for frequent cage cleaning that increases animal stress. For IVCs, cage changes every 14 days and 60 air changes per hour are considered optimal.

o    Isolators: flexible-film or semi-rigid wall isolators. Used to maintain germ-free or gnotobiotic animals.

* + Effects of social housing, group size, density and housing conditions are to be considered. There many variables (species, strain, phenotype, age, gender, space characteristics, in-cage structures, enrichment etc.). There are no conclusive data on the effect on animal welfare and study results.
	+ Husbandry procedures are associated with several behavioral and physiologic effects in rodents that can alter metabolism and study results. For example, cage-changing frequency, staff gender, staff wearing fragrance, presence of other male animals, animal handling methods, chemicals used for cleaning, cage autoclaving times and type, sterility of food and bedding, type of bedding and nesting material, water quality and treatment, equipment, material and personnel entrance barriers and SOPs,

o    After cage-changes there is a minimum 48-96 h disruption of normal cage activity.

QUESTIONS

1. Which sentence is CORRECT regarding micro-isolation caging systems?

a. Individually ventilated cages require frequent cleaning

b. Static caging produces high noise and vibration

c. Static caging results in rapid elevation of ammonia levels

d. Ventilated cages are not appropriate for barrier housing

2. Which of the following is NOT true for polyphenylsulfone cages?

a. Damaged cages may release Bisphenol A and S, which are endocrine disruptors

b. Their amber/yellow coloration results in 35% less light penetration

c. They can be autoclaved in up to 143°C

d. They have a 3-year lifespan

3. True/False: All aspects of animal husbandry shall be described in detail in publications, as these may affect experimental outcomes.

4. Which of the procedures below may have an effect on animal physiology and/or behavior:

a. Cage changing frequency

b. Personnel wearing fragrance

c. Water quality and treatments

d. All of the above

ANSWERS

1. c

2. a

3. True

4. d

# **Pritchett-Corning. Environmental Complexity and Research Outcomes, pp. 239-251**

Domain 4: Animal Care

SUMMARY: Environmental complexity is defined by comparisons, with barren environment on one end of the spectrum and environment in the wild on the other. Basic environmental complexities can be grouped into 6 categories: item, social, nutritional, spatial, control, and sensory. Beyond basic categories, there are 3 arbitrary, classifications: standard, super-, and semi-naturalistic environments. Standard is a normal research environment, super enrichment is a larger space with items rotated often and allows for same sex groupings, and semi-naturalistic is much larger, often outdoors, allowing for mixed sex groupings. Standardization across these groups is nearly impossible, but enrichment can significantly affect study results. Therefore, researchers should be encouraged to describe the housing environment in great detail to allow others to duplicate work.

Positive effects of more complex environments on play and aggressive behavior, cognition, and immune responses have been established. Examples of increasing complexity include training animals for cooperation during blood collection to reduce stress, providing materials that are related to the natural environment, and providing items that allow animals to express highly motivated behavior patterns. The goal of increasing complexity in the research setting can be achieved by cooperation and consultation between all groups involved in laboratory animal care.

QUESTIONS

1. Which category of basic environmental complexity would include a running wheel?

a. Item

b. Social

c. Spatial

d. Control

2. Which category of basic environmental complexity would include essential oils?

a. Items

b. Spatial

c. Sensory

d. Control

3. Which of the 3 arbitrary categories listed as beyond basic would the following scenario fit into: 3 female rhesus macaques socially housed in a large, indoor play cage with items rotated several times weekly?

a. Standard environment

b. Super-environment

c. Semi-naturalistic environment

d. All of the above

ANSWERS

1. a

2. c

3. b

# [**Whittaker**](https://academic.oup.com/ilarjournal/search-results?f_Authors=Alexandra+L+Whittaker) **and Hickman. The Impact of Social and Behavioral Factors on Reproducibility in Terrestrial Vertebrate Models, pp. 252-269**

Domain 3: Research

SUMMARY: Animal models continue to be a critical part of biomedical research. The reliability and validity of animal models are important to translating the findings into medicine. While animal research can provide considerable uniformity among studies through controlled genetics, environments, and experimental design, there are still several social and behavioral factors that may confound research and affect the reproducibility and translatability of these results. This article described some of the factors that can influence research results in animal models. While not all of these factors can be controlled for and their effects on specific research may be unknown, an awareness of these different factors and their potential influence on study results is crucial, as is careful study design that could limit the impact of these factors.

Genetics: Animal genetics are essential to determining the appropriate model for study. The use of genetically modified and transgenic animals can remove many confounding variables in a study. Since genotype affects phenotype, it is important to do genetic testing to maintain quality assurance and ensure that genetic drift or spontaneous mutations are not affecting the phenotype.

Age and Development: For most animals, behavioral expression changes of the animal’s lifetime, as do cognition and neural processing. Epigenetic factors can also affect cognition and neural development. Careful selection and standardization of subject age is crucial to good study design. All factors of the environment should be evaluated for their potential effect on research outcomes.

Sex: Males and females are different and consideration of sex on research results is critical. Studies should be designed so that the effects in males and females are equally evaluated. Exposure to members of the opposite sex can also affect certain species behaviors and physiology, so separation of animals by sex may be needed, depending on the scientific question.

Personality: Individual animal behavior is shaped by many factors that cannot always be controlled for in a scientific setting and/or across different laboratories. Providing as much detail as possible on the techniques for animal handling and technical approach in publications will be important to increase reproducibility in these studies.

Human-Animal Interaction: The effect of the sex of the experimenter and the interaction between the animal and its human handler can vary among species. The human experimenter has been recognized as a potential confounding factor in neuroscience studies and studies with significant behavioral components. Consideration of who will handle the animals and how they interact and handle the animals is important to minimizing variables.

Interspecies Interaction: The effect of interspecies interactions is not always well characterized. In general it is recommended that animals be separated by species unless interspecies interaction is central to the research question.

Pain and Distress: The presence of pain or distress can influence biological function and if unrelieved, can negatively affect research studies. Minimizing pain can be accomplished through use of analgesic medications, development, and refinement of ways to recognize and assess pain in different species, and by utilizing new, less invasive experimental techniques that may reduce the need for painful procedures. Recognizing and alleviating animal distress is more challenging but there are a growing number of assays being developed for different lab animal species to help address this issue.

Social Factors: These include the effect of housing factors on different species, such as isolation housing, single housing, and group housing. If group housing is utilized, it is important to remember that while negative social interactions can adversely affect animal welfare and research, social interactions can also help animals modulate stressful experiences and build resilience. The interaction of the neural, endocrine, and immune systems is well recognized and studies on social buffering tend to focus on the HPA axis. There are many factors and practicalities that affect how animals may be housed and the type of social interactions they can have. It’s important to recognize that all of these may influence research results.

QUESTIONS

1. Social buffering has been documented in which of the following species?

a. Nonhuman primates, rodents, and birds

b. Rodents, fish, and birds

c. Nonhuman primates only

d. Dogs, cats, and rodents

2. Which of the following is the most common CNS inhibitory neurotransmitter, a deficit of which can result in anxiety, seizures, and insomnia?

a. Glutamate

b. Dopamine

c. Serotonin

d. GABA

3. The ARRIVE guidelines are a checklist of recommendations to improve the reporting of research involving animals. What does ARRIVE stand for?

a. Accurate Research and Reporting in Vertebrate Experimentation

b. Animal Research: Reporting of In Vivo Experiments

c. Animal Research: Requirements for Indicating Variability in Experimentation

d. Accurate Reporting Requirements and Information for Valid Experiments

4. What is the term used to describe the social sharing of emotions among rodents and may increase the probability of similar behavior in others and quick adaptation to challenge?

a. Empathy

b. Resilience

c. Vulnerability

d. Emotional contagion

ANSWERS

1. a

2. d

3. b

4. d

# **Lieggi et al. The Influence of Behavioral, Social, and Environmental Factors on Reproducibility and Replicability in Aquatic Animal Models, pp. 270-288**

Domain 4

Primary Species **–** Zebrafish (*Danio rerio*)*, Xenopus laevis, X. tropicalis*

Secondary Species **–** African Clawed Frog (*Xenopus laevis* and *X. tropicalis*)

SUMMARY: Reproducibility in scientific research is a fundamental component of quality studies. In biomedical research ethical treatment of animals in pursuit of scientific objectives is equally important. The aim of this article is to review many important variables that must be considered in two of the most common aquatic animal models: *Danio rerio* and *Xenopus laevis* / *X.* *tropicalis* with a focus on behavior and tank design.

ZEBRAFISH

Behavior: Zebrafish are omnivorous and opportunistic feeders living in shallow freshwater ponds. They are primarily visual hunters preferring red colors and feed in all water strata. Larvae use mechanosensory systems to forage in the dark. Zebrafish are highly attuned to predation and there are substantial differences in behavior as a result of predation pressure between wild and domesticated zebrafish. Other factors that affect behavior include olfaction in response to pheromones released during reproduction and other pheromones released during injury. Reproductive behavior may be affected by phenotypic traits and social hierarchies and fish behavioral adjustments based on these variables may influence breeding success, clutch size, and fertilization rates. Zebrafish are a shoaling species which gives them an advantage in the wild in foraging efficiency and predator avoidance. Shoaling is the aggregation of the same species whereas schooling refers to a pattern of movement as a group. Stress or novelty will cause fish to school but once acclimated they revert to shoaling. There are many examples of environmental factors that will affect this behavior. Even temporarily isolating a fish from the group for a study treatment as is common in pharmacologic studies for example, will cause stress and alter behavior of the group once the individual fish is returned potentially affecting an observed phenotype. Social isolation and pair-housing are both stressful and should be avoided when possible. Socially isolating larval zebrafish has lasting effects into adulthood. Cortisol levels may be elevated or decreased with chronic stress so should be interpreted carefully.

Sex and strain differences can also influence behavior and there is high individual variation in behaviors. Health status also affects behavior. A classic example is the effect of *Pseudoloma neurophilia* infection and the effect on startle response habituation and it also alters shoaling behavior.

Housing: While there is no established ideal tank size or design, consideration to color preferences and other environmental variables should be considered based on the goals of the study. Zebrafish are similar to rodents in that they prefer some structure in their environment over empty tanks which can induce anxiety behaviors. Environmental enrichment is overall beneficial to zebrafish well-being, but social hierarchies should also be taken into account. For example, structures may give subordinates a place to hide but may also give a dominant fish something to defend thereby increasing aggression. Other external variables to consider are lighting, abruptness of lighting on/off, noise, and vibration.

Crowding in zebrafish tanks can cause a variety of alterations including altered sexual development, plasma cortisol levels, and interference with cardiac tissue regeneration. Guidelines on optimal densities are variable in the literature and there is not a set standard. N.B. The “Guide for the Care and Use of Laboratory Animals” recommends 5 fish/L with the caveat that this recommendation could change with new research.

XENOPUS

*X. laevis* and *X. tropicalis* are the two most commonly used amphibians in research. *X. laevis* is the South-African Clawed Frog is a highly adaptable and invasive species. *X. tropicalis* is geographically more restricted and has narrower environmental needs. Xenopus have relatively poor eyesight but well-developed olfactory, auditory, mechanoreceptor and electroreceptor (lateral line) systems. Anything that affects the sensory hairs of the lateral line system can affect behavior. For example, streptomycin given to *X. laevis* tadpoles.

Behavior: Tadpoles and adults should be considered separately. Tadpoles form conspecific schools whereas adults do not demonstrate schooling behavior. Adults are territorial and form hierarchies. Competition for food within a tank may negatively affect growth of subordinate frogs. An optimal stocking density has not been defined but environmental and social factors should be considered. Xenopus are indiscriminate feeders preferring live food but adapt readily to pelleted food in the laboratory. They can regurgitate their stomachs and use their forelimbs to wipe away inadvertent ingestion of something toxic or unpalatable including orally administered experimental compounds. Feeding time incites a frenzy which can result in bite wounds therefore, it is better to feed fewer food items more frequently throughout the day. A defense mechanism is to use their strong hind legs to flee which means they can easily jump out of shallow tanks or transport carriers. Their slime layer which contains toxins, antimicrobials and antivirals also offers a method of defense so damage to this layer or chronic stress can alter this defense. Chronic stress leads to elevated cortisol and subsequent immune dysfunction. Stress can also cause regurgitation resulting in poor nutrition. As with all aquatic animals handling can be a significant source of stress. Xenopus are nocturnal so light-cycle should always be considered when designing studies. Their skin can change in response to hormones and background colors. Tank color or opacity can affect growth, hormone levels, and behavior. Water flow can affect behavior, and this differs between stage of development. Water flow can also be a stressor as can excessive vibrations. Environmental enrichment: hiding places are recommended to decrease stress and aggression but there are not many studies on this topic.

Conclusions: Controlled conditions are important for generating conclusions, reducing confounding factors, making comparisons between laboratories, and allowing replicability but they may miss important differences when factors that can alter behavior are not considered, and they are rarely standardized across laboratories. Some of the factors that may easily be overlooked are strain, sex, age, group dynamics, tank environment, external environment such as climate, light conditions, and acoustics. When designing a study, it is important to consider all these variables plus other details unique to a species and then to report in detail the laboratory conditions under which the animals are kept.

QUESTIONS

1. T or F: Laboratory raised zebrafish are uniform in their behaviors.

2. Which of the following does not appear to alter zebrafish behavior?

a. Stocking density

b. Plastic plants in the tank

c. The sex of the animal caretaker

d. The presence of other fish in the tank

3. T or F: Adult Xenopus demonstrate schooling behavior in a normal setting?

4. Which of the following is a sign that a tank containing Xenopus may be under chronic stress?

a. Regurgitation of the stomach by some of the frogs

b. Bites wounds on arms and legs of some individuals

c. Various body sizes of individuals

d. All of the above

 ANSWERS

1. False: There is considerable individual variation in zebrafish behavior including those raised in captivity.

2. c: In one study there were no observable differences in behavior when zebrafish were cared for by a male vs female caretaker.

3. False: tadpoles demonstrate this behavior but not adults

4. d: these all may be signs of chronic stress in Xenopus

# **Franklin and Ericsson. Complex Microbiota in Laboratory Rodents: Management Considerations, pp. 289-297**

Domain 4

Primary Species: Mouse (*Mus musculus*)

SUMMARY: There are a number of factors that result in subtle changes in microbiota, which likely create great variation in GM of contemporary rodent research colonies. Disease models may show variation in disease expression over time even within the same laboratory.  Questions are raised whether variation in GM contributes to variability and reproducibility of rodent models. Recommended to focus on experimental design and report conditions so that experiments can be replicated.

Must show that transfer GM results in transfer of phenotype. This can be done in number of ways,

1. Co-housing of mice with desired phenotype with isogenic mice that lack the phenotype- relies on coprophagy for GM transfer. However, complex GMs are often stable and colonization resistance can prevent key members of GM from colonizing intended recipients. Can result in hybridization of GM and not in phenotype change – so caution when characterizing GM in recipient mice.

2.  Bedding- feces transfer or soiled bedding from mice with desired phenotype to one that lacks phenotype; relies on coprophagy, therefore colonization of key bacterial species may not be achieved in recipient mice.

3.  Fecal transplantation to antibiotic- treated mice. Recipient mice pre-treated with broad- spectrum antibiotic mixes to reduce endogenous microbiota; then gavage with slurry prepared from donor or other intestinal samples with desired GM. Transfer can be limited, especially when attempting to transfer GM of low richness to recipients initially colonized by high richness GM.

4.  Fecal transplantation to Gnotobiotic mice- requires specialized facilities, more expensive. Can be gold std which complex microbiota transfer can be achieved; transfer may not be complete, especially if obligate anaerobes lost during preparation of transfer material.

5.  Derivation by Embryo Transfer. Derived by ET using surrogate dams with the desired microbiota, resulting in neonates exposed to vaginal microbiota during delivery, oral microbiota during neonatal care and cutaneous and mammary microbiota during nursing. Also, shared maternal bloodstream while in utero. However, more expensive and labour intensive; second gold standard technique.

Consideration in Choice of Transfer Technique: Depends on study goals and resources (monetary and physical plant)

If changes in any GM- modulating factors are anticipated, periodic fecal banking should be considered à collected fecal samples and storing at -80C (need to further study cryopreservation techniques).

GM can change during rederivation when attempting to eliminate pathogens. Therefore, need to consider that GM can vary with facilities/ producer’s rooms and between genotypes; therefore, the source of the mouse has a greater influence than genotype on GM composition of inbred mice.

MMRRC at University of Missouri has developed 4 colonies of outbred mice that harbor GMs obtained from the 4 major suppliers/ producers in the United States. GM of these colonies is monitored quarterly and remained stable over 20 generations. There is an annual ‘refreshing’ with GM known to mirror the original composition to avoid small drifts- amenable to be used in controlled studies. Colonies can be used in the reconstitution methods mentioned above and can be supplemented with specific target bacteria to assess their role in a ‘standardized’ complex GM background.

3 approaches to consider optimizing translatability:

* Humanization of GM through fecal or intestinal content transplants.
* Use of wild or pet store mice that are exposed to much more diverse environment that correlates with richer GM.
* Comparison of functionality of complex communities instead of comparison of species composition.

Some limitations of complex GM studies include:

* Many contemporary studies rely on targeted amplicon sequencing using 16S rRNA gene to determine bacterial phylogeny, but whole bacterial genome shotgun sequencing improves speciation and bacterial strain assignment. Data must eventually be linked to functional assessment like meta-transcriptomics or metabolomics.
* Sequence databases for annotation (that only maps to genus, family, or order) so there is inability to cultivate many intestinal bacteria that have emerging interest.
* There is need for bioinformatic tools for study of complex interactions of bacteria as well as between microbiome data and metabolomics data.
* Temporal studies of intestinal microbiota are from feces, which does not reflect higher levels of GIT like jejunum, ileum, and cecum.
* Need to incorporate role of metagenome that includes other microbes like viruses, fungi, and parasites.

QUESTIONS

1. Which of the following can result in subtle changes in microbiota?
	1. Producer vendor
	2. Diet
	3. Housing enclosure
	4. Genetics and sex
	5. b & c
	6. All of the above
2. MMRRC at University of Missouri has developed 4 colonies of outbred mice that harbor GMs obtained from the 4 major suppliers/ producers in the United States. Which of the following is true?
	1. GM of these colonies is monitored quarterly and remained stable over 20 generations.
	2. There is an annual ‘refreshing’ with GM known to mirror the original composition to avoid small drifts- amenable to be used in controlled studies.
	3. GM of these colonies is monitored bi-annually and remained stable over 10 generations.
	4. a & b
	5. a & c

ANSWERS:
1. f

2. d