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Review

Outside the Box: Working With Wildlife in Biocontainment

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Abstract

Research with captive wildlife in Animal Biosafety Level 2 (ABSL2) and 3 (ABSL3) facilities is becoming increasingly necessary as emerging and re-emerging diseases involving wildlife have increasing impacts on human, animal, and environmental health. Utilizing wildlife species in a research facility often requires outside the box thinking with specialized knowledge, practices, facilities, and equipment. The USGS National Wildlife Health Center (NWHC) houses an ABSL3 facility dedicated to understanding wildlife diseases and developing tools to mitigate their impacts on animal and human health. This review presents considerations for utilizing captive wildlife for infectious disease studies, including, husbandry, animal welfare, veterinary care, and biosafety. Examples are drawn from primary literature review and collective 40-year experience of the NWHC. Working with wildlife in ABSL2 and ABSL3 facilities differs from laboratory animals in that typical laboratory housing systems, husbandry practices, and biosafety practices are not designed for work with wildlife. This requires thoughtful adaptation of standard equipment and practices, invention of customized solutions and development of appropriate enrichment plans using the natural history of the species and the microbiological characteristics of introduced and native pathogens. Ultimately, this task requires critical risk assessment, understanding of the physical and psychological needs of diverse species, creativity, innovation, and flexibility. Finally, continual reassessment and improvement are imperative in this constantly changing specialty area of infectious disease and environmental hazard research.

Key words: ABSL2, ABSL3, animal welfare, biohazard containment, biosafety, captive wildlife, enrichment, husbandry

INTRODUCTION

Emerging infectious diseases, defined as novel or known infectious diseases increasing in incidence within a specific location or population, and environmental contaminants pose global and profound threats to human, animal, and environmental health.[1,2] The rise of emerging infectious diseases demonstrates the dynamic relationship among pathogens, hosts, and their environment.[3,4] Over 60% of emerging infectious diseases are zoonotic, and over 70% of those zoonoses have a wildlife nexus,[3] including highly pathogenic avian influenza virus (HPAI), sylvatic plague, Lyme disease, anthrax, severe acute respiratory syndrome and severe acute respiratory syndrome-CoV-2. These diseases typically increase burdens on public health systems, negatively impact the world economy, cause declines and extinctions of animal species, and increase loss of ecological integrity.[5,6] The potential global impact of a wildlife-associated pathogen on human health is exemplified by the over 35 million people currently infected with human immunodeficiency virus, which is reported to have originated from a simian (primate) virus.[7] Likewise, negative effects of emerging and resurging diseases on agriculture, food safety and security, wildlife health, and human health in Southeast Asia have resulted from

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outbreaks of HPAI.[8,9] There are also several newly described pathogens and diseases that have resulted in wildlife population declines and global extinctions. *Batrachochytrium dendrobatidis*, a fungus that causes cutaneous infection of amphibians, is linked to global declines of amphibian populations,[10] and *Pseudogymnoascus destructans*, the etiologic agent of white-nose syndrome, has caused precipitous declines in the abundance of North American hibernating bat species.[11] Such large-scale losses of animal species and biodiversity subsequently jeopardize the ecosystems on which all life depends.[12,13] Of particular concern are novel emerging infectious diseases of wildlife origin because they are difficult to anticipate; potentially devastating to wildlife populations, public health, and the economy; and challenging to manage and have the potential to have long-term ecological ripple effects. The prevalence of emerging diseases and pathogens of wildlife origin is increasing globally at alarming rates in both incidence and by geographic location, which can be largely attributed to the driving forces of globalization, an increasing human population, and environmental and climate change.[3,5,6]

This rise in emerging infectious diseases of wildlife origin has intensified the need for research to predict, identify, respond, manage, and recover from these emerging disease events. One framework used by the USGS National Wildlife Health Center (NWHC) that provides methods for attacking this complex problem includes risk assessment at the human-livestock-wildlife interface, predictive and forecasting modeling, pathogen discovery and characterization, disease surveillance, development of management tools, and methods to assess effectiveness of actions. Interdisciplinary research that combines multiple disciplines from both natural and social research themes is, therefore, required. Laboratory-based, experimental approaches are a critical component of this interdisciplinary research and are needed to test hypotheses and to study causal relationships (ie, how and why disease outbreaks occur). Controlled laboratory-based experiments are also necessary to fulfill Koch's postulates for newly discovered pathogens, understand pathogenesis, contribute to the development and validation of diagnostic methods, understand host range and species susceptibility, elucidate routes of pathogen transmission and other epidemiological parameters, and develop management tools such as vaccines (Figure 1).[14] Experimental studies should not be conducted in isolation and should be combined with descriptive studies, field experiments, and other techniques such as mathematical modeling as described above to fill knowledge gaps and design intervention strategies. Randomized controlled experimental trials are particularly vital because they provide results that are considered relatively robust on the hierarchy of evidence, especially because we are often faced with a paucity of data on novel diseases and surveys of free-living wildlife populations can be challenging to conduct due to logistical constraints.[15]

There are very few established experimental animal models for work with wildlife species; consequently, research studies require working directly with the wildlife taxa of concern, resulting in some unique challenges in an experimental setting. This article highlights these challenges and the differences with working with nontraditional species in this environment, including biosafety, facilities, animal care and husbandry, animal procurement, quarantine and veterinary care, and personnel and training. We use examples and case studies from the NWHC and primary literature to illustrate the unique considerations that researchers with wildlife species in Animal Biosafety Level 2 (ABSL2) and 3 biocontainment facilities have to make to characterize emerging pathogens and to develop tools to prevent and control diseases in wildlife populations that impact human, animal, and environmental health.

WILDLIFE HUSBANDRY

Wildlife Housing

Housing systems of captive wildlife require adaptable approaches tailored to meet the needs of the animal, research, and facilities, and this necessitates thinking outside the box. There are few housing systems that are standard for wildlife because taxa vary in size, shape, and general health needs.[16,17] Standard domestic or laboratory animal caging may not be suitable even for closely related taxon. Furthermore, recommendations for livestock housing may be inappropriate for wildlife, and guidelines on housing, such as appropriate room or pen size, may not be readily available for many wildlife species.[16] In general, housing for wildlife follows standards developed by the "Guide for the Care and Use of Laboratory Animals" (the Guide).[18] However, the Guide was not written as a manual for wildlife containment. Unlike common laboratory animals, the Guide does not provide specifics on cage size or minimum space recommendations for prairie dogs (*Cynomys* spp.), but it does provide specifics for guinea pigs (*Cavia porcellus*).[18] Although these taxa differ in natural history and feeding requirements, using the minimum space requirement provided by the Guide for similar sized taxa provides a starting point. Furthermore, it is difficult to specify minimum cage size for research animals, because animal strain, group size, locomotion, age, reproductive conditions, and familiarity with each other all impact the development of housing systems.[16,19] For example, standard non-human primate housing posed concerns for marmosets at ABSL3 because the mesh on the outside of the cage was considered too large, allowing marmosets the means to escape and ultimately forcing the construction of custom caging.[20] This is true for wildlife in captivity as well. At the NWHC, custom caging was built with additional floor space for studies of rabies virus in common vampire bats (*Desmodus rotundus*) (Figure 2B). This was important because this species is unique among bats, in that, in addition to flying, it also runs and jumps on the ground.[21] Poor welfare conditions caused by inadequate housing not mimicking a taxon's natural history have been associated with physiological, immunological, and behavioral changes in animals that can ultimately affect reliability of the scientific data.[20,22–25] Finding safe and functional housing systems combines multi-faceted knowledge of the taxon and the disease system and will require nontraditional approaches combined with outside the box solutions.

Containment caging, which controls air flow and prevents escape of aerosolized pathogens, is typically designed for smaller common laboratory species such as mice, rats, ferrets, and rabbits used in biocontainment studies.[26,27] Some of these common laboratory species can be used to model certain diseases in wild taxa (eg, mice for development of plague vaccines for wildlife).[28,29] Similar wildlife species may be housed in containment caging in some cases (eg, deer mice [*Peromyscus maniculatus*] or meadow voles [*Microtus pennsylvanicus*] housed in mouse containment caging), but these types of containment caging could be stressful for other wildlife taxa due to the loud air handling systems, low humidity, or inability to accommodate appropriate social groups.[30] Similarly, there is also containment caging designed for agronomically important species such as

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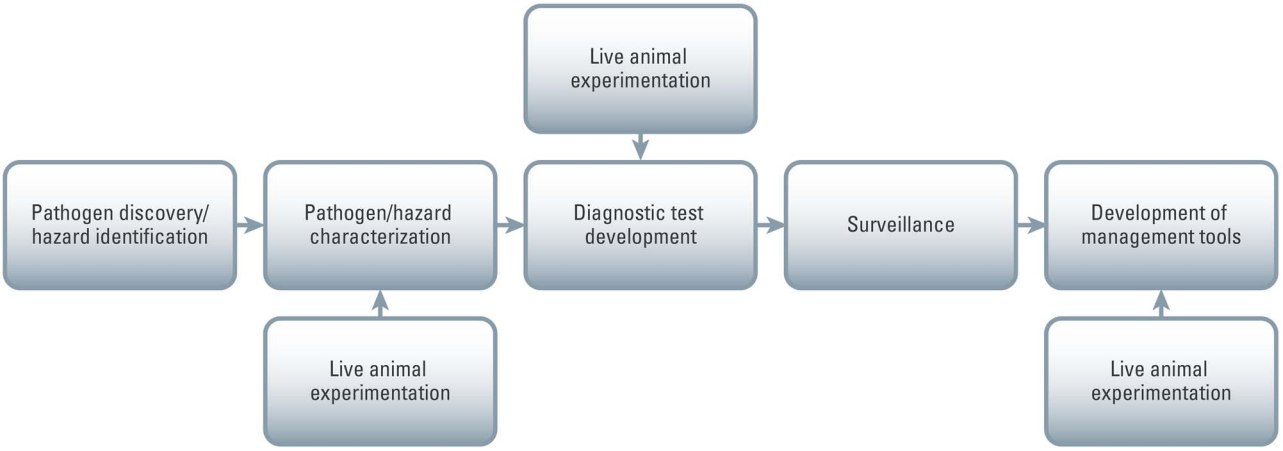

Figure 1: The research pipeline from pathogen discovery/hazard identification to the development of management tools, and the multiple areas of contribution of live animal experimental work.

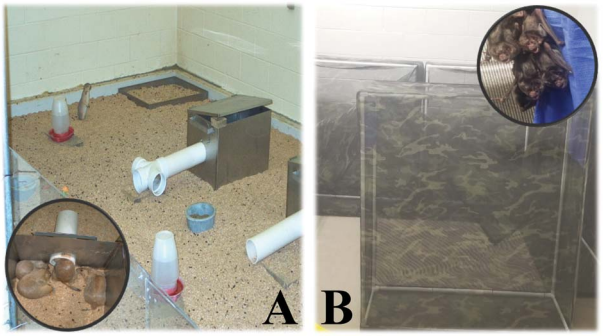

Figure 2: Custom-made housing is often required for highly social wildlife species. (A) Shows black-tailed prairie dogs (*Cynomys ludovicianus*) housed on the floor in a custom made "burrow" system, designed to allow the animals to display their normal social structure. This housing was used for challenge studies with *Yersinia pestis*, ABSL-3 pathogen, because individuals of this highly social species become stressed, depressed, and anorexic when housed individually (T. Rocke, personal communication). (B) Shows custom made cages for common vampire bats (*Desmodus rotundus*), with room for flight, as well as floor space for crawling and jumping, a unique behavior of this species, among bats. The dimensions of the cage were designed so that all areas of the cage were reachable by personnel, to facilitate individual monitoring of disease after challenge with rabies virus (ABSL-2 Pathogen).

poultry and swine and these can be altered to appropriately house wildlife. For instance, poultry isolator cages have been modified to house different avian taxa for avian influenza viruses and other avian disease studies, including passerines, shorebirds, and raptors.[31–33] For HPAI and other select agents and toxins, containment caging may be required by the Federal Select Agent Program.[34–36]

Although there are companies that can custom design containment caging for different species, this is not always a viable option when working with wildlife due to the size of the animal, specialized environmental requirements, social needs, or cost. To balance animal welfare with biosafety, it may be necessary to house animals in a manner that more closely mimics their natural environment, wherein the animal holding room serves as primary containment. When using the room as primary containment, creative caging setups and enrichment programs can increase the likelihood of having calm animals that are easier to handle, mitigating biosafety concerns from bites and scratches. This ultimately improves the quality of the research study with fewer animals being removed from a study due to health or behavioral issues. As seen in Figures 2A and 2B, custom housing systems can be designed to meet the physical and social needs of wildlife species, such as for the black-tailed prairie dog (*Cynomys ludovicianus*) and the common vampire bat (*D rotundus*). Both were inoculated with risk group 3 (RG3) and RG2 pathogens, respectively, in these housing systems, using additional personal protective equipment to mitigate biohazard risks. Similarly, wild-caught mallards (*Anas platyrhynchos*) were housed in pens using the room as primary containment prior to infection with HPAI (RG3) at NWHC (Figure 3). Such arrangements, as seen in Figure 3, allow for normal social interactions, species-typical behaviors such as swimming and bathing, and minimized pododermatitis, a common problem in captive waterfowl.[37] Just prior to infection with HPAI, these birds were housed in pairs in poultry isolator cages. Even if using containment caging, modifications can be made to enrich the environment and provide hiding areas, which can help to reduce stress and therefore risk injury to those handling the animals.[38] In the example above, additional nutritional and physical enrichment was offered in the cages, and isolator cages were modified to allow a mesh divider to separate the ducks in case of social incompatibility. These strategies are similar to those used when housing larger domestic animals in containment.[17]

Finally, indoor housing in a biocontainment facility presents some additional challenges. Wildlife are not domesticated, and their fear of humans makes them more of a risk to personnel and themselves.[16] For example, white-tailed deer (*Odocoileus virginianus*) have been used in biocontainment facilities for investigations of infectious pathogens such as bluetongue virus (RG3).[39] Deer are easily startled by humans, which increases the risk of slips or falls that can result in contusions, lacerations, or fractures within their containment space. To mitigate the risk of slipping on the floor, a biocontainment facility at Colorado State University housed deer on a mixture of sand and epoxy, which reduced injury and resulted in relatively normal hoof wear.[40] As illustrated, working with wildlife in ABSL2 and ABSL3 facilities requires balancing animal welfare and biosafety and often requires creative solutions.

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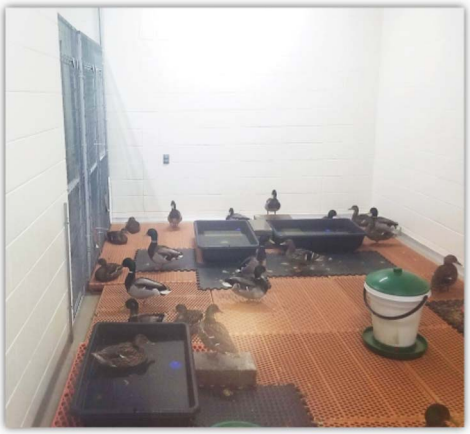

Figure 3: Housing for mallard ducks (*Anas platyrhynchos*) using the room as the primary containment barrier. This type of housing allows for social enrichment, physical enrichment (pools), and promotes good health and welfare by providing multiple surfaces to reduce the incidence of pododermatitis, which is common in captive waterfowl.[37] It did not meet requirements of the federal select agent program for highly pathogenic avian influenza, so later portions of the study were conducted in poultry biocontainment caging.

Figure 4: (A) Shows the first version of a hibernation chamber used to house torpid bats at the USGS National Wildlife Health Center. This was a florist cooler (SRC Refrigeration, Sterling Heights, MI) modified with a gravity-fed watering system (1), a reptarium cage (Apogee, No Longer Manufactured), a surgical towel for roosting (2), and tubs of water to increase humidity (3), in addition to the timed water mister. (B) Shows a later version of the hibernation chamber, which consists of a custom-made environmental chamber (Percival, Perry, IA), which maintained tight control of temperature and humidity and featured a viewing window (1), a surgical towel (HT1 sensor, Sensorpush, Brooklyn, NY) for roosting (2), a simple gravity-fed chicken waterer (3), a Sensorpush probe to send temperature or humidity failure alerts to the animal care team (4), and red backlighting to allow better visualization of bats through the viewing window without opening the chamber (5).

Social Housing

The concept of social housing for social species is not new and it not unique to wildlife.[18] However, the natural social structure of wildlife species is often complex and can be more difficult to mimic in biocontainment than that of standard laboratory animals. In general, social housing creates an environment that supports growth and development for social species. Within the biocontainment facility, it may be desirable to house animals singly or in unnatural group arrangements to accommodate easier pathogen containment. However, this may result in stress associated with abnormal social interactions and could negatively affect the outcome of the study. Social deprivation of gregarious animals can lead to significant changes in brain chemistry, behavior, hormones, metabolism, and immunity.[41–43] These physiological effects may be present without outward behavioral signs.[42] Additionally, size of groups and established groups of animals need to be considered.[44,45] Acquiring insufficient numbers of individuals to form natural groups or lack of available space in captivity may prevent optimal social group composition and size. For example, prairie dogs (*Cynomys* spp.) have a complex social structure in which territorial familial groups (coteries) live in individual burrow systems within larger colonies.[46] In studies of plague (*Yersinia pestis*) in prairie dogs at the NWHC, large groups were co-housed on the floor of a vivarium room with a burrow system composed of stainless-steel bins and tubes (Figure 2A), which allowed them to maintain their normal social groups and interactions,[47] whereas individually housed prairie dogs became anorexic and depressed due to social isolation (T. Rocke, personal communication). As demonstrated in these examples, keeping animals in appropriate social groupings and with the required space and complexity to allow individuals to choose to spend time together or apart is likely to be the most important welfare consideration.[48] For all captive animals, housing must meet the biological and psychological needs of the species; for wildlife species with complex social structures, this may require additional research and creativity to achieve within ABSL2 or 3 facilities.

Temperature, Humidity, and Light

When formulating housing conditions for wildlife in biocontainment, the natural history of the taxa should be the foundation of all decisions. At times, this will dictate extreme temperature, humidity, or lighting. Achieving and maintaining proper temperature and humidity at the room level is critical for wildlife health. Temperature is especially important for poikilotherms that are acclimatizing to captivity.[30,49] For example, warmer conditions than the natural environment during the initial transfer to captivity resulted in high mortality in sardines (*Sardina pilchardus*)[50] and cane toads (*Rhinella marina*).[51] Both transport and housing temperatures should mimic the natural setting as closely as possible. For instance, when wild-caught fish, aquatic frogs, or newts arrive at a facility, the water in the new habitat should be the same temperature as their transport water. To achieve this, the transport container can be floated in the water of the new habitat to slowly acclimate the animals (P. Johnson, personal communication).

For most laboratory animals, a humidity of 50% is sufficient,[18,52] whereas some wildlife taxa require much higher humidity (>70%).[21] In facilities where humidity is difficult to control within an acceptable range, dehumidification or humidification devices (misters, foggers, and household humidifiers) may be installed. For example, captive studies of white-nose syndrome in insectivorous bats caused by *P destructans* require bats to be in hibernation in controlled environments mimicking the low temperature and high humidity conditions of their hibernacula.[53] To overcome this unique requirement, the NWHC developed specialized hibernation chambers. The first prototypes were modified florist coolers (Figure 4A; SRC Refrigeration, Sterling Heights, MI, USA).[53] Later versions became more specialized and were custom-built environmental chambers (Percival Scientific, Perry, IA, USA) (Figure 4B).[54]

Lighting is important to animal behavior and physiology across a broad range of wildlife taxa, and attempts should be

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made to mimic natural setting.[55–57] Animals in their natural environment use the sun as a source of radiation, warmth, and light, but it also provides species with vitamin D.[49] Vitamin D directly affects the growth and development of bones and neuromuscular function and is important to reproductive, immune, and cardiovascular health.[58] A deficiency in vitamin D caused by an insufficient amount or improper spectrum of light in captive reptiles and other sunlight-sensitive wildlife has been linked to inadequate musculoskeletal development, embryonic death, pathological fractures, and respiratory failure.[59–61] Interestingly, dietary supplementation of vitamin D proved to be inadequate in green iguanas, emphasizing the importance of adequate and appropriate spectrum light.[62]

The photoperiod, intensity, and spectral composition of the light are all important features of lighting for research animals in captivity.[63] Detrimental health effects from inadequate lighting are likely more important for wildlife due to moving from the natural environment to captivity. Specifically, incorrect photoperiod or light changes (eg, not mimicking natural history of the taxon) have been correlated with a negative effect on reproduction, behavior, growth, hormones, and survival, which ultimately altered research outcomes.[64] A sudden increase in light exposure triggered mating behavior and severe aggression in a group of laboratory-housed white-tailed prairie dogs (*Cynomys leucurus*) (Falendysz, unpublished data). Photoperiod and light should not only mimic natural environments for wildlife in containment but should also be considered when infecting wildlife with microbes or parasites. For example, parasites such as trematodes in frogs and toads are subject to daily rhythms in the within-host environment, resulting from clock-control of host activities, including immune responses.[65] *Ribeiroia ondatrae*, known to cause limb malformations in many species of frogs and newts, releases its cercariae at night to mimic the noncturnal activity pattern of tadpoles.[65] Lastly, in some taxa of wildlife, specific photoperiod and light composition is necessary for captive breeding.[66]

Noise, Vibration, and Odors

Some wildlife taxa, though seemingly adaptable to typical laboratory environments, may be highly sensitive to subtle odors, high-pitched sounds, infrared light, ultraviolet light, or magnetic fields that cannot be detected by humans or other animals. For example, common vampire bats (*D rotundus*), like other bats, have excellent high-frequency hearing. Anything above approximately 17 kHz is imperceptible to most humans, whereas a vampire bat can hear up to 113 kHz.[67] In a captive colony of big brown bats (*Eptesicus fuscus*), noise and vibration from nearby construction were temporally associated with an outbreak of stress-induced anorexia and subsequent hepatic lipidosis, even causing death in some animals.[68] Finally, in biocontainment facilities, it is important to consider how the disinfectants, containment housing, and facility infrastructure may be perceived by each species.[69,70]

Seasonality

Seasonality of wildlife is an important consideration in housing that is not necessary for laboratory-raised animals. When transferring wildlife to containment, understanding the daily and seasonal variations in behavior and physiological functions that are naturally influenced by the geographical location and climate of the species can decrease stress. Wildlife taxa can be categorized in relation to their activity patterns: diurnal, nocturnal, crepuscular (active at dawn and dusk), matutinal (dawn and morning), verspertine (dusk and night), and cathemeral (when activity is distributed roughly evenly throughout the 24-hour cycle).[71,72] It is important to acknowledge differences within activity budgets, and these in turn can differ per individual.[48] Ignoring these variations could lead to chronic stress. Seasonality needs to be considered when evaluating the health of wildlife in captivity as well. For instance, captive ruffs (*Calidris pugnax*) and red knots (*Calidris canutus rufa*) have strong seasonal weight fluctuations in captivity associated with weight gain for migration and breeding.[30,73] If semi-naturalistic conditions are maintained in captivity (eg, if the animals are exposed to natural day length), then they may continue to experience seasonal weight changes that are not due to overfeeding or to long-term stressor exposure.

Diet and Feeding

In general, when designing a diet for wildlife, qualitative information on natural feeding habits and quantitative data on food nutrient composition and utilization will provide an optimal diet for captive wildlife. There are very few references that provide these 2 components from a single source.[74,75] Some key factors to consider when preparing a diet for wildlife are food habits in the wild, including when and where taxa prefer to eat (eg, nocturnal in a cave); digestive system of taxa with special attention to lips, dentition, and type of digestive system (eg, simple, complex); studies on natural diet composition (eg, isotope analysis); phylogenetics to determine if there is a close domesticated relative; age; food delivery within the housing system (eg, containers, temperature of food, mode of feeding); and how food consumption could alter disease dynamics.[74,76–78] Using premade diets sold by laboratory companies and consulting a nutritionist can provide basic details that will assist in providing a balanced diet. Additional sources include textbooks[77,79] and the Association of Zoos and Aquarium's Nutrition Advisory Group.[80] Finally, newly housed animals will likely need to adapt to the new environment before they are comfortable consuming food.[76,77] For example, big brown bats (*E fuscus*) and little brown bats (*Myotis lucifugus*) in nature forage on flying insects.[21,81] However, in biocontainment facilities they are commonly fed mealworms (*Tenebrio molitor*), because it is not practical or safe to have flying insects in containment.[21,82] For bats to recognize that mealworms are food, they require days to weeks of training to eat non-volant insects from a dish.[21]

Delivery of water to wildlife in containment caging can be troublesome and needs special consideration because it differs from common methods used in laboratory research. For example, most common laboratory animal models such as mice or hamsters have commercially available rodent water bottles. Water delivery may be different for taxa that do not drink from a bottle or from a bowl such as hummingbirds, which drink from flowers.[83,84] Several characteristics render hummingbirds as an interesting species for the study of wildlife disease ecology experimentally.[85] They are known to carry *Encephalitozoon hellem* that causes keratoconjunctivitis, sinusitis, and, uncommonly, systemic disease in humans.[85,86] Similarly, sandgrouse (*Pterocles spp*.), which are important model animals for understanding dessert dwellers, drink by soaking up water by specialized belly feathers.[87] Lastly, the timing of when water is delivered and volume of water consumed can vary seasonally.[88]

MINIMIZING STRESS

Regardless of the animal biosafety level and taxon, all animal containment facilities need to consider minimizing and mitigating stress. Unlike typical laboratory research animals, wildlife is not bred for living in captive conditions and will likely find

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many aspects of the captive environment stressful. Knowledge of each taxon's natural history is a key component in decreasing all forms of stress that can pose negative impacts on the animals' health and the quality of research outputs. Furthermore, stressed animals will also be more reactive and thus more potentially dangerous when infected with a pathogen.[17] It is not uncommon for wildlife that are used in experiments to be captured and transported directly to a biocontainment facility (see procurement section) and the conditions of captivity (eg, confinement, artificial lighting, changes in diet, noise, and the presence of and handling by humans) are stimuli that can activate the stress response systems.[49,63] For example, the extreme change from natural to artificial light can cause health concerns, or even death, in wildlife, by keeping the animal in a constant state of excitement.[49]

Enrichment

Enrichment programs are essential to a balanced husbandry program, because stimulating and responsive environments are basic required conditions to meet an animal's physical, physiological, and psychological needs.[89] Thus, they are essential for wildlife in containment as vital components in the husbandry program. Natural wildlife environments are complex, and that complexity should exist in their research environment within scientific, financial, and logistical reason. Designing an enrichment program for wildlife requires creativity, and the needs are situational and taxa dependent.[16,90] Furthermore, achieving adequate welfare for wildlife is more difficult due to lack of knowledge of normal behavior and physiological needs and the fact that some animals are wild caught and not accustomed to a captive situation.[91–93] When developing an enrichment plan for wildlife, it is crucial to determine what normal behavior observed in the wild is desired within the research setting. For example, aggression in nature is normal but will need to be avoided in a research setting. Five categories of enrichment should be considered to aid in positive natural behaviors for wildlife: nutritional, sensory, social, physical, and environmental.[89,94] Not all species will have a need for each category, and some may have multiple for the same category. For instance, at the NWHC, an enrichment plan is developed for each species that attempts to include 1–2 options for each category (Supplementary Table 1). Enrichment programs should consider the sensory capabilities of the specific wildlife species. Programs should also consider the dominant senses used in their natural setting and use care not to use stimuli that are overly stressful.[95,96] These types of programs are likely to result in the greatest benefits for animal welfare. For example, for studies testing an experimental rabies vaccine, NWHC provided environmental enrichment to insectivorous bats by adding folded surgical towels to provide crevices that mimic roosts (Figure 4B). Additionally, ladders were provided to stimulate natural climbing behaviors (physical enrichment). Sounds that might be found in their native environment were also played, such as rustling leaves, flowing rivers, and insect calls, to stimulate their sense of hearing (auditory enrichment), which is one of the primary ways that a bat senses its environment.[97] Enrichment programs can also help to reduce stress.[98,99] For example, the natural environmental sounds mentioned above were played during cleaning procedures for the insectivorous bats to help to decrease stress associated with this unnatural activity (NWHC, unpublished data). Similarly, in studies investigating rabies vaccine delivery in wild raccoons (*Procyon lotor*), researchers modified standard raccoon caging to include a covered plywood nest box for privacy and sleeping and an open wire cage used for feeding and exercise.[100] These modes of enrichment mimicked the natural environments raccoons use during these activities. Finally, enrichment programs that serve to decrease stress, as evidenced by reduced circulating cortisol,[101,102] improve the welfare of the wildlife[103,104] but also data quality and safety to the handler.[105]

Development of an enrichment program for wildlife in biocontainment adds a second layer of complexity because all enrichment items must be free from pathogens, parasites, and bacteria and must be able to be decontaminated.[106] These requirements result in elimination of some items or habitat modifications commonly used in agricultural or zoo animal husbandry programs. Creative modifications are typically necessary for commercially made products containing materials that cannot be chemically disinfected or are unable to withstand high temperatures (eg, wood, non-food grade plastics). In other settings, it is common to provide natural elements from wild habitats, but these too must be free of pathogens and other pests before being brought into containment. The NWHC collects pinecones and leaves each fall, then dries and autoclaves them prior to use in big brown bat habitats to provide environmental, auditory, and oral enrichment. Because they cannot be repeatedly disinfected, these items are discarded with the other contaminated waste after use. Also, due to the risk of the pathogen or toxin exposure to the workers, extra caution needs to be considered with materials that are sharp or could become sharp if accidently broken. It is best practice to eliminate the use of glass in a habitat to reduce the risk of injury and potential biohazard exposure to personnel,[106] even though glass can withstand high temperatures and is easily disinfected.

Handling and Restraint

Handling and restraint of wildlife in a containment facility requires the ability to recognize the animal's psychological condition, which may include aversion, confusion, fear, discomfort, or pain. As discussed previously, wild animals are part of food webs, so humans are either predators or prey depending on the taxa. Preconditioning or training of wildlife takes time and, in some circumstances, is not recommended, especially for research studies where results are to reflect natural circumstances. In other cases, training may provide additional access for observation, such as training animals to present a specific body part that could be necessary for sampling in a long-term infectious disease study.[16] In general, guidelines for working safely with experimental wildlife include understanding the species-typical behavior when handling, using appropriate occupational health precautions, understanding how the animal communicates, using appropriate restraint techniques and equipment, and identifying an animal that may act unpredictably.[16,107,108] Specialized personal protective equipment and handling equipment vary with pathogen and taxa but should always be worn when physical contact is necessary with wildlife. Some examples of specialized handling and restraint equipment include nitrile/latex gloves, puncture-resistant gloves, various nets, Y poles, animal control poles, plastic face shields, forceps, plexiglass restrainers, and plastic restrainers. Specialized equipment may not be available commercially but can be developed in-house with a little innovation. For example, we developed a neoprene bat restrainer for safe and reduced-stress blood collection by taking advantage of the bats' natural preference for small, confined

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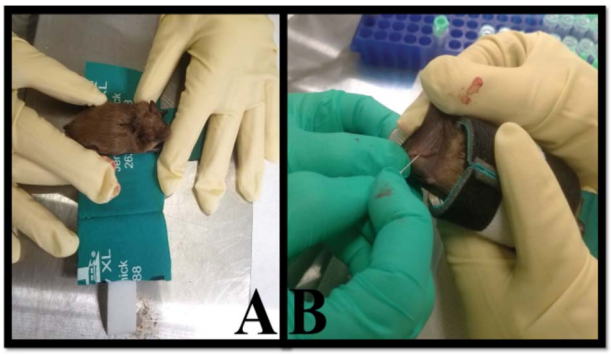

Figure 5: A custom-designed restraint for blood collection in *Eptesicus fuscus* (S. Smith, personal communication). (A) A Neoprene-insulated can holder and Velcro strap were used to make a restraint device, taking advantage of the bats' preference for tight, dark spaces to make them feel more secure during restraint. The bat restrainer is wearing leather and Kevlar gloves under long-cuffed latex gloves. (B) With the bat in the restraint device, the uropatagial (interfemoral) vein is accessible and blood is collected using a needle and capillary tube, with a metal thimble used to protect the finger of the blood collector.

spaces (crevices) (S. Smith, personal communication; Figure 5). Further, starting with calmer, less stressed animals by providing enriched, species-appropriate husbandry will reduce the risk of injury and laboratory-acquired infection.[105]

Restraint of wildlife species also has the potential for significant health impacts on the animal. Two concerns are capture myopathy and capture shock. Capture shock occurs due to a combination of metabolic acidosis, hyperkalemia, and ischemic necrosis worsened by a severe adrenergic response and can result in acute cardiovascular collapse and death.[109,110] Capture myopathy is often a deadly outcome to capture and restraint in wildlife species. Extreme exertion, typically from a flight response, causes lysis of skeletal or cardiac myocytes, leading to acute cardiac failure, acute or subacute myoglobinuric nephrosis, ruptured muscles (commonly the gastrocnemius muscle), or a delayed peracute syndrome that causes death from atrial fibrillation upon a second stressor in the following days or weeks.[109,110] Capture myopathy is most common in ungulates, especially cervids and antelope species,[110] cetaceans,[111–113] and long-legged wading birds[110,114,115] but occurs in other mammals and birds such as carnivores.[116–118] For wildlife, it may be safer for the animal and the handler to administer anxiolytic or sedative drugs for physical restraint to minimize stress associated with handling.[119,120]

PROCUREMENT AND VETERINARY CARE

Procurement

Procurement of wildlife species for captive studies is often a distinct process from that typically used for more common laboratory animals. The most common sources of animals are capture from native or non-native habitats, captive breeding colonies, and commercial wildlife breeders for the pet trade or for hunting. Among other factors, the source of the animals likely has a significant impact on their risk for infection with various pathogens and will thus play a large role in the risks to humans and other animals in their laboratory environment. Captive breeding colonies and commercial wildlife producers are likely to be sources with the least risks.[16]

Using animals captured from the wild creates some limitations. It will take significantly more resources to acquire animals, which may limit the number of animals that can be obtained and will make it difficult to add more animals to the study, should some animals not be suitable for any reason (ie, health status, maladaptation to captivity, etc). Species biology and natural history may limit when animals are available or the specific sex and age classes that are available. For example, big brown bat (*E fuscus*) females congregate in maternity colonies with their weaned offspring during the spring and summer, but males are solitary at this time and are more dispersed on the landscape, making them more difficult to capture. However, all age and gender classes congregate in the fall and are found together in hibernacula in the winter.[121] Capture of any native wildlife within the United States for research purposes will require one or more permits issued by the state or federal wildlife management agency responsible for that species. If importing animals from other countries, import permits may be required by the Centers for Disease Control and Prevention,[122] United States Fish and Wildlife Services,[123] the United States Department of Agriculture,[124] and/or the Convention on International Trade in Endangered Species of Wild Fauna and Flora.[125] Animals captured from the wild also pose the highest biosafety risk because their history is less defined and there is a higher potential for the animals to be naturally infected with pathogens. Despite these limitations, wild-caught animals can often provide results that are more reflective of what may occur in nature compared with domesticated or captive-bred animal models.

Captive breeding facilities used for the exotic pet trade and other purposes may also be suitable sources of research animals. One advantage is that it may be possible to request specific disease screening prior to shipment. For example, mallard ducks (*A platyrhynchos*) are frequently bred for pets, egg production, or hunting sports. Many of these producers participate in the National Poultry Improvement Plan and thus have more robust biosafety, biosecurity, and pathogen surveillance plans.[126] Establishing an in-house breeding colony of a specific species may be required when large quantities of individuals are needed or when a specific age or disease exposure status is required.[127,128] For example, rabies virus exposure is difficult to accurately assess antemortem in bats, even using serology. If a study requires bats that are naïve to rabies virus, a properly quarantined breeding colony may be the most suitable option.[129]

For all animals, transportation also requires significant planning. Unique to species captured from the wild, the infection status of the animals may be unknown at the time of shipment. When it is reasonable to assume an animal may be infected with a zoonotic or agricultural agent, precautions should be taken to isolate these animals during transport or to treat them before shipping. For example, black-tailed prairie dogs (*C ludovicianus*) captured from areas with endemic circulation of sylvatic plague (*Y pestis*) were dusted with topical parasiticide before transport to eliminate fleas and reduce the risk of transmission.[130] In some cases (eg, common vampire bats, *D rotundus*), the animals themselves may be considered as vectors of RG3 agents and regulated as such.[122] When there is a risk of aerosolized pathogens of concern, HEPA-filtered containment can be designed and constructed using various materials, including those designed to transport immunodeficient laboratory animals.[131]

Quarantine

Quarantine and testing procedures should be carefully designed for wildlife studies. Reproducible data is facilitated by standardization, or characterization, of the health status of the animals used in research.[132] Unlike animals bred in captivity, it is often

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impractical to assess the health and infection status of wild animals before transporting them from their site of capture.[133] Therefore, this kind of assessment is typically made once animals are within the captive environment, during some period of quarantine and acclimation. The health and infection status of animals can have direct impacts on the data produced from the study, the risks to humans working with animals, and the risks to other animals that are housed in the same facility or local environment.[132,134,135] Therefore, determination of the quarantine and testing procedures for a specific study is best made by discussion among the research team, biosafety personnel, the facility veterinarian, and the animal facility manager.

The first step is a risk assessment that considers the species, age, sex, geographic location, and other factors to determine the likelihood that various pathogens are present in the animals and the risks to humans, other research animals, and research outcomes.[134] Once it is determined which pathogens are desirable to exclude, quarantine and testing procedures can be developed as for typical laboratory species. It should be assumed that some parasitism is likely in most wildlife species. Further, 70% of emerging zoonoses come from wildlife,[3] so it may be prudent to assume the risks of zoonoses in wildlife are higher and/or less well characterized than in domestic animals. A third difference for wildlife compared with traditional laboratory or agricultural animals is that there may be a higher likelihood that antemortem diagnostic testing is unavailable or unreliable for the detection of some pathogens. Examples of infectious diseases of wildlife that are difficult to determine the antemortem infection status are rabies in bats,[129] cryptosporidiosis in reptiles,[136,137] and mycobacteriosis in birds.[137] When a single antemortem test is unreliable, an alternative approach to reduce risk is to quarantine the animals for suitable periods to wait for disease to become clinical or to use repeated diagnostic tests to increase the likelihood of detection. For example, Rivera[135] recommends quarantine of 90 days or more for reptiles that are wild caught or when the health history of the source colony is unknown. Even with quarantine periods of 90 days, adenovirus, arenavirus, *Chlamydophila pneumoniae*, and *Cryptosporidium sp*. infections in snakes may not be clinical, and thus additional testing for these pathogens would be required to exclude them.[138] Quarantine and testing procedures should be included in resulting publications to ensure data reproducibility across studies, because both source and quarantine procedures have significant effects on the microbiological status of research animals.[139,140] Finally, if it is decided to use generalized assessments of health such as serum chemistry or complete blood counts to determine that an animal is healthy enough to participate in a study, it is possible that there are no reported normal reference intervals or that these reference intervals will be based on a small number of animals.[141] This makes interpretation of the results challenging. A well-conceived quarantine and disease testing plan will improve biosafety for humans and other animals in the facility and will result in more consistent, reproducible data.

Wildlife Veterinary Care

Veterinary care and oversight of wildlife in research presents unique challenges. One of the challenges of working with wildlife as research animals is a lack of studies of pharmacokinetics and pharmacodynamics in most wildlife species, requiring extrapolation from known taxa to calculate drug dosages.[142] With the breadth of species used in wildlife research, the evolutionary relatedness of any well-studied species could be phylogenetically distant. For example, specific drug dosages are known for most anesthetic and analgesic drugs in laboratory rodent species (eg, rats, mice, and guinea pigs), and considerable differences are recognized between these taxa.[143,144] However, when treating a species without specific pharmacological data, one must extrapolate from other species. To the authors' knowledge, peer-reviewed studies of analgesics used in bat species are limited to a single pharmacokinetic study of meloxicam in Malayan flying foxes[145] and 2 case reports reporting the use of meloxicam and butorphanol in several species.[146,147] Therefore, the suggestion of textbooks on bat analgesia and anesthesia can only reference anecdotal doses or those used in various rodent species[21,148,149] despite the very wide evolutionary distance between rodents and bats.[150] This type of broad extrapolation has proven to be inappropriate in some cases, specifically in birds and reptiles. Although it was once assumed that all bird families responded similarly to opioid pain medications, it is now well known that analgesic efficacy is different between raptors and psittacines.[108,151,152] Likewise, analgesic efficacy of various opioids is variable among snakes, chelonians, and lizards.[153] To fill these knowledge gaps, analgesic and antimicrobial pharmacology are active areas of research in the field of zoological medicine.[153,154] It is imperative that a literature search be performed frequently to stay current on the use of these drugs in various in taxa. However, it is likely that information will have to be generalized across distantly related species. In addition to peer-reviewed literature, guides and texts written for wildlife rehabilitators or zoo and aquaria may contain drug dosages, anesthetic plans, and suggested routes of administration.[107,155–158] A detailed understanding of the differences in physiology between different taxa can help guide decisions about how best to extrapolate drug dosages.[159] Lastly, it is important to remember to balance the effect of handling on stress to the animal and risk to the handler with the benefit of the drug or treatment used. Chronic stress response leads to decreased immune function and healing,[160–162] fight or flight responses to handling can lead to self-injury or injury to the handler,[107] and capture shock or capture myopathy are difficult to treat and are often deadly.[107,110] In general, wildlife species are less amenable to frequent handling for administration of medicines, so long-acting medicines or those compounded into a food item are often necessary for good treatment success.[154,163–165] Further research and documentation of treatments used on wildlife patients are needed to provide veterinarians precise and proper dosages for many species.

ADMINISTRATIVE AND BIOSAFETY CONSIDERATIONS

Training

Ensuring appropriate training for work with captive wildlife in research, as required by the Animal Welfare Act and Public Health Service policy, is more complicated compared with laboratory animals.[124,166] The training must cover husbandry and biology of the species to be used, with special attention to the natural history of the taxa. Training on proper handling and restraint for each species is important to promote good animal welfare, useful scientific outcomes, and adequate biosafety.[105] Even species that are within the same family or are the same size can respond differently to the same restraint.[16,107] Unlike laboratory species, training specific to each wildlife taxon is often not available from outside sources. Therefore, many institutions

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rely on 1-on-1 training from experienced individuals, including the investigators. In some cases, training can be developed that pertains to a broader taxon, such as training to work with bats[167] or reptiles,[168] but it may also be appropriate to combine this with didactic training on the unique needs of individual species. Because it is often not possible or practical to obtain wild animals solely for the purpose of training, it may be more appropriate to use animals that are intended for the study for training purposes. Additionally, another way to accomplish training is to use an alternate species followed by the trainer or veterinarian observing the first attempt at performing a new skill with the target wildlife species. For example, African clawed frogs (*Xenopus laevis*) could be used for training on procedures that will be utilized with other anurans.[169] Redundancy in training is key, especially for activities that have higher safety risk.[106] As the biosafety risk increases, entities often require a minimum number of observed hours with a more experienced person to ensure the individual has mastered procedures before allowed to work with a high-risk pathogen. In summary, for research or animal care staff to be successful in their jobs, specific, tailored training is vital.

Biosafety for Captive Wildlife

To determine the appropriate animal biosafety level for a research study, the first step is to assess the types of pathogens or biological toxins that will be administered to an animal; however, when working with wild-caught animals, one must consider not only the pathogens or toxins that will be introduced experimentally but also the pathogens or toxins an animal might naturally harbor. To determine what pathogens may be present in a wild population, factors such as the geographic location of the animal, season in which the animal is to be captured, age of the animal, as well as others should be used to assess the likelihood of various pathogens. Furthermore, for all pathogens and toxins, both introduced and naturally occurring, factors such as route of transmission, stability in the environment, susceptibility to disinfectants, severity of disease, and infectious dose are considered in the risk assessment. However, the naturally occurring pathogens may also pose more of a threat (ie, increased risk) and can dictate a higher biosafety level than the introduced experimental pathogen or toxin. For example, in a study utilizing wild-caught herring gulls (*Larus argentatus*) to investigate the pathogenicity of a suspected bacterial pathogen of birds that is not zoonotic and is therefore suitable for ABSL1 containment, the gulls were housed at ABSL2 because gull species are a known reservoir of avian influenza,[170] which is a greater risk to personnel handling the gulls than the introduced pathogen (C. Smith, personal communication). Because avian influenza viruses can vary in prevalence in wild bird populations depending on the season, host species, age, and geographic locations,[171] strategies can be developed to target populations used in experimental studies that may have a lower risk of avian influenza virus infection. Similarly, considering natural pathogens along with experimentally introduced pathogens is especially important when selecting an appropriate disinfectant for a study; the selected disinfectant should be effective against both types of pathogens, even those that affect animals other than humans. For example, *Cryptosporidium serpentis* is an environmentally hardy parasite that can be carried and shed by many species of snakes and can cause disease in some individual snakes.[172] In a study in which corn snakes (*Pantherophis guttatus*) were inoculated with a python nidovirus, the animal room was decontaminated with 6% hydrogen peroxide at the end of the study,[173] even though less harsh disinfects such as ethanol or sodium hypochlorite are effective against enveloped viruses such as nidoviruses[106] (NWHC, unpublished data).

Animals, especially wildlife, originating from other states or countries may carry pathogens that are not native to the locality of the research facility, and a release into the environment could be costly to domestic animals and wildlife.[174–177] To protect the environment from the biological hazards present in an animal containment room (both introduced and pre-existing pathogens), facility enhancements can be considered depending on the risk assessment, including HEPA-filtered exhaust,[178,179] redundant exhaust fans, or shower out facilities at the room level or facility level.[34–36,175] Additionally, the assessment should include whether an effluent decontamination system is needed.[17,105,180] Permits and local waste regulations should also be examined to determine if an effluent decontamination system is required. This is especially important if the room is used as primary containment and cannot ensure that all animal waste that enters the drain has been decontaminated. These facility enhancements can provide maximum flexibility for various types of husbandry and pathogen containment needs.[17,105,180]

CONCLUSIONS

There is an increasing need to research diseases in wildlife to characterize, diagnose, and manage emerging threats to human, animal, and environmental health. Wildlife studies in biocontainment facilities rarely fit into typical laboratory husbandry and biosafety protocols, requiring novel approaches and thinking outside the box. Critical assessments using primary literature, alternative sources of information, and subject matter expertise is required to develop husbandry, biosafety, and veterinary management plans consistent with the natural history of the species and the pathogen being studied. Creativity and innovation are often required to achieve the goals of providing animal welfare, biosafety, and quality data with wildlife in experimental settings. Working with wildlife in biocontainment requires special skills and training, continuous learning, and excellent communication across all levels of the program. When traditional approaches to husbandry and biocontainment do not work for a given taxa or pathogen, researchers and laboratory animal scientists will have to find creative solutions. Publication of methods for use of wildlife taxa in biocontainment should be encouraged to avoid unnecessarily duplicating these development efforts. And, as for all laboratory animal science, all aspects of the program should be continually reassessed and improved as new information and techniques become available.

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Supplementary Data

Supplementary materials are available at *ILAR Journal* online.

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