IACUC Issues Associated with Amphibian Research

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Abstract

Numerous species of amphibians are frequently utilized as animal models in biomedical research. Despite their relatively common occurrence as laboratory animals, the regulatory guidelines that institutional animal care and use committees (IACUCs) must employ provide little in the way of written standards for ectothermic animals. Yet, as vertebrates, laboratory amphibians are covered by the National Research Council *Guide for the Care and Use of Laboratory Animals* and the Public Health Service (PHS) Policy for federally funded research. This article focuses on three issues that are relevant to IACUC oversight of the use of amphibians in research: (1) recommended educational requirements of investigators and animal care staff engaged in research with amphibians, (2) zoonoses and other issues of occupational health importance, and (3) indicators of stress and disease. Addressing these issues should enable investigators, IACUCs, and animal care staff to meet the regulatory expectations of the PHS and accrediting bodies such as the Association for Assessment and Accreditation of Laboratory Animal Care International.

Key Words: amphibian; education; IACUC; occupational; research; stress; zoonosis

Introduction

A number of amphibian species are commonly used in biomedical research. Of the three orders of amphibia (anura, caudata, and gymnophiona), the most commonly used in research are anurans, particularly the genera *Xenopus, Rana*, and *Bufo*. "Caudates" are less frequently seen in laboratory research settings, with the axolotl salamander as a relatively frequent example. However, the potentially wide variety of amphibian species used in research, combined with their varying husbandry, care, and medical requirements, can present institutional animal care and use committees (IACUCs[1]) with challenges related to meeting an array of novel research requirements. This difficulty is further compounded by a relative paucity of regulations and standards-oriented documents for ectothermic species (particularly the less common species) used in biomedical research.

In this article, we first outline the current regulatory requirements and guidelines in the United States. Then, because not all IACUCs members have extensive research or veterinary medical expertise associated with laboratory amphibians, we discuss the following three topics: (1) recommended educational requirements of investigators and animal care staff engaged in research with amphibians, (2) potential zoonoses and other issues of occupational health and safety importance, and (3) indicators of stress and disease. Finally, we address some of the more prominent issues facing animal care and use committees when dealing with laboratory amphibians.

Regulatory Requirements and Guidelines

IACUCs are responsible for ensuring an institution's compliance with federal regulations that govern the conduct of animals used in research, teaching, and testing (AWA 2002; PHS 2002). Although ectotherms (including amphibians) are specifically excluded from the Animal Welfare Act (AWA[1]) and thus are not subject to the inspection regimen of the US Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS[1]), all research and teaching activities funded by the Public Health Service (PHS[1]) require review and oversight by the IACUC. The PHS Policy on Humane Care and Use of Laboratory Animals (PHS Policy[1]) specifically endorses application of the

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[1]Abbreviations used in this article: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; AWA, Animal Welfare Act; CITES, Convention on International Trade of Endangered Species of Wild Fauna and Flora; IACUC, institutional animal care and use committee; MS-222, tricaine methanesulfonate; NRC, National Research Council; NRC *Guide*, *Guide for the Care and Use of Laboratory Animals*; OLAW, Office of Laboratory Animal Welfare; PHS, Public Health Service; PHS Policy, The Public Health Service Policy on Humane Care and Use of Laboratory Animals; SOP, standard operating procedure; USDA-APHIS, United States Department of Agriculture-Animal and Plant Health Inspection Service.

standards delineated in the National Research Council (NRC[1]) *Guide for the Care and Use of Laboratory Animals* (NRC *Guide*[1]), which technically covers all vertebrates used in nonagricultural research, teaching, or testing (PHS 2002). The exception is for agricultural animals used for agricultural teaching and research, which are covered by the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*, published by the Federation of Animal Science Societies and commonly known as the "Ag Guide" (FASS 1999).

The NRC *Guide* is a useful document with regard to general standards for the IACUC's oversight of all research animals; however, specific standards for amphibian care and husbandry are not covered in its scope. Indeed, in the *Guide*, it is specifically stated that "because of the large number of nontraditional species and their varied requirements, this Guide cannot provide husbandry details appropriate to all such species" (NRC 1996, p. 5). It is also stated, however, that several scientific organizations have developed guides for the care of such nontraditional laboratory animals (NRC 1996, p. 5), and that "users, IACUCs, veterinarians, and producers use professional judgment in making specific decisions regarding animal care and use" (NRC 1996, p. 3). The Office of Laboratory Animal Welfare (OLAW[1]), which develops and provides oversight of PHS policy, concurs by indicating that "the PHS Policy is intentionally broad in scope and does not prescribe specifics about the care and use of any species, assigning that task to the IACUC and allowing for professional judgment" (Potkay et al. 1997, p. 48). The OLAW authors continue, however, with a declaration supporting application of the principles delineated in the NRC *Guide* by stating, "Many of the principles embodied in the *Guide*, although not specifically addressing cold-blooded vertebrates, generally can be adapted to animal care and use programs for various kinds of amphibians . . . " (Potkay et al. 1997, p. 48). Thus, although OLAW does not mandate absolute standards for laboratory animal care and does allow for professional judgment, it is clear that adherence to NRC *Guide* principles is an expectation. Consequently, most institutions accept the NRC *Guide* as a standards document, regardless of the funding source. Borski and Hodson (2003) have explored similar issues surrounding fish research in a previous issue of *ILAR Journal*.

In addition to these federal regulations, it is important to note that importation, transportation, or transfer of some species (particularly endangered species) may be governed by the US Fish and Wildlife Service and by the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES[1]). Several dendrobatid frogs are listed in Appendix II of CITES; Appendix II aims to control the trade of some species not necessarily threatened with extinction to avoid utilization that is incompatible with their survival (CITES 2006). Finally, the most important accrediting body for laboratory animal research programs uses the NRC *Guide* as the standards document for all of its programmatic evaluations. Institutions endeavoring to pursue accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC[1]) must adhere to the standards stipulated in the NRC *Guide*.

Recommended Educational Requirements of Investigators and Animal Care Staff Engaged in Research with Amphibians

Research institutions must apply to amphibians the regulations that govern the use of animals. The IACUC has the responsibility and authority for overseeing the care and use of all species. For an IACUC that has not previously dealt with many amphibian projects, resources are available that can help address the new questions and challenges. The attending veterinarian is an appropriate source for information about the appropriate care and humane use of amphibians. He or she may have the expertise necessary to guide the IACUC, or may develop it as needed for individual species. The IACUC may also consult another professional who has expertise with amphibians. Other resources include published references on the husbandry, veterinary care, and biomethodology of amphibians. A list of some of these resources is provided in the appendix at the end of this article, and additional Internet resources are provided elsewhere in this issue (Nolan and Smith 2007).

Published resources, especially for the less frequently used amphibians, are not as extensive as those for the more commonly used animals. Nevertheless, several excellent references are available for the more commonly used amphibians, the most prevalent of which is the African clawed frog, *Xenopus laevis*. Its use is popular with developmental biologists and in cellular and molecular biology research (O'Rourke and Schultz 2002). Indeed, the number of relatively recent publications describing the care and use of *Xenopus* attests to the frequent use of this species, particularly among academic laboratory animal care programs (Crawshaw 1992b; Godfrey and Sanders 2004; Green 2002; Gresens 2004; O'Rourke and Schultz 2002; Schultz and Dawson 2003; St. Claire et al. 2005; Tuttle et al. 2006). One excellent resource that addresses the use of amphibians in general is *Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research*, published by the American Society of Ichthyologists and Herpetologists, The Herpetologists' League, and the Society for the Study of Amphibians and Reptiles (ASIH/HL/SSAR 2004).

One challenge the IACUC faces is working with researchers who are not familiar with animal research regulations and are not aware that their projects require IACUC approval. In the academic environment especially, departments that may be more likely to use amphibians often have less contact with the IACUC. Departments that deal with research or teaching in ecology, wildlife, or conservation are frequently not as familiar with the IACUC and animal research regulations as are biomedical or molecular biology departments. The institution should be proactive and educate these researchers by tasking the appropriate department heads to disseminate information to their faculty and staff. An IACUC website can provide information specifically relevant for these researchers to guide them in complying with the regulations.

The typical IACUC protocol form is designed to provide all of the information required by the regulations. A general form, however, may not address all of the concerns relevant to amphibian use, especially in a field study situation, and the IACUC may need to consider issues that are not the subject of questions on the form. An IACUC that is evaluating research using amphibians faces the same animal use concerns as always, but using amphibians both expands the usual concerns and adds novel matters of importance. Overall, the following topics remain relevant and must be addressed: institutional policies and responsibilities; animal environment, housing, and management; veterinary medical care; and physical plant. In addition, it may be necessary to reconsider the following areas when making decisions regarding the use of amphibians: new dimensions in monitoring and oversight; personnel issues; husbandry; population management; animal procurement and transportation; preventive medicine; and guidelines for surgery, pain, analgesia, anesthesia, and euthanasia. Examples of questions to ask include the following: Who will provide veterinary care for animals housed at a field site? Will captured animals be housed and then released? If so, where will they be released?

The IACUC must verify the appropriate physical environment, behavioral management, husbandry, and population management of captive amphibians.

One challenge the IACUC faces is working with the researcher who prefers to provide the husbandry for his/her animals. Although this arrangement may have advantages if the researcher has expertise in housing and managing the particular species, a lack of direct oversight can be a disadvantage. In this situation, the IACUC has the responsibility for oversight, and must carefully assess the adequacy of care. Opportunities for IACUC assessment include semiannual site inspections and program reviews as well as researcher-provided documentation of daily care. The IACUC may ask a researcher to submit written standard operating procedures (SOPs[1]) that describe the housing structure, enrichment, husbandry practices, environmental monitoring, and reproductive methods, if applicable. The IACUC can assist the researcher in writing these documents by providing templates or examples inasmuch as many researchers are unfamiliar with animal care regulations and unaware of what information is required.

The institution has the option of obtaining the necessary expertise, and in some situations the animal care staff provides the daily care and husbandry. Specialized training may be required to maintain the housing appropriately, monitor the environmental parameters, master unfamiliar equipment, and develop new skills. The institution may need to invest in personnel and training, but the advantage is more direct oversight and control of the care.

The use of amphibians in research commonly occurs via a field study. Field studies may involve only noninvasive observation or may include capture and manipulation of free-ranging amphibians. In general, according to the NRC *Guide*, "Investigators conducting field studies with animals should assure their IACUC that collection of specimens or invasive procedures will comply with state and federal regulations and this *Guide*" (NRC 1996, p. 5). However, the use of animals in a field situation incurs a specific set of concerns for the IACUC.

The IACUC responsibility for monitoring animal care and use applies to field station facilities. The PHS regulations require that the IACUC, or subcommittee, "inspect the animal facilities and activity areas at least once every six months" (PHS 2002). Because research using amphibians frequently occurs in the field or at remote field station satellite locations, semiannual site visits are problematic. For field sites, the NRC *Guide* provides flexibility and states that ". . . some of the recommendations in this volume are not applicable to field conditions . . ." (NRC 1996, p. 5). Although it does not specify which recommendations are not applicable, the ". . . basic principles of humane care and use apply . . ." (NRC 1996, p. 5), According to OLAW, "IACUCs must know where field studies will be located, what procedures will be involved, and be sufficiently familiar with the nature of the habitat to assess the potential impact of the animal subjects" (OLAW/OER/NIH 2006).

Field stations require more direct oversight. According to the PHS Policy, all animal housing facilities must be inspected semiannually, including satellite facilities (OLAW/OER/NIH 2006). At institutions that support a significant amount of research using wild-caught species, it becomes extremely time consuming to visit multiple remotely located field stations. Currently, OLAW has no recommended alternatives to semiannual site inspections for field stations.

Researchers should be advised that they will be required to cooperate with the IACUC to schedule inspections. If animals are housed at a location seasonally, researchers should notify the IACUC both when animals are moved into the field station and when they are removed. Unlike laboratory animal housing facilities, field stations frequently do not have personnel trained in laboratory animal care, regulations, or IACUC policies. Thus, it becomes the researchers' responsibility to provide animal housing and husbandry information, preferably in SOP format, to the IACUC during semiannual inspections.

Procurement of amphibians is more complex than with more common laboratory species. Several common amphibian species used in research and teaching, such as *Rana* spp. and *Xenopus* spp. frogs, can be purchased from commercial vendors. However, these vendors differ from those of common laboratory rodent species. Routine, extensive health monitoring is not performed, so animals' health status is unknown. Although commercial venders sell animals that they breed in house, they may also capture and sell free-ranging animals. The researcher, animal care staff, and attending veterinarian should be aware of the source because it may affect the animals' health status. Researchers frequently capture free-ranging amphibians because the species is not commercially available or because they are studying a particular aspect of the animal in its habitat. Again, the health status is unknown, and infections are possible. Researchers sometimes breed amphibians in house or obtain them from commercial vendors as embryos or tadpoles. Embryonic amphibians are not covered by the PHS Policy, but they are covered after hatching into larval stages (OLAW/OER/NIH 2006).

The capture of free-ranging amphibians involves many new issues for the IACUC. There are many methods of capturing free-ranging amphibians, and the IACUC should ensure that the proposed methods are appropriate and minimize the risk of injury to the amphibian. If unattended traps are used, the IACUC should consider how often the traps are checked and whether food and/or water are available in the trap, whether the trap is sheltered from environmental stresses such as sunlight, and whether the captive animal is protected from predation. Capture of free-ranging animals may result in unintentional capture of other species, so the proposed capture methods should minimize the risks of this situation occurring. In addition, the researchers should have a plan for the release or euthanasia of any by-catch individuals (ASIH/HL/SSAR 2004).

The IACUC must be aware of laws governing the collection and/or transport of wild-caught amphibians. Local, state, and federal permits are required for collection of amphibians. Collection of endangered or threatened species requires special collection permits from the US Fish and Wildlife Service (ESA 1973). International studies introduce the requirements of other countries and may involve CITES. In the NRC *Guide*, it is stated that researchers should assure their IACUC that collection of specimens complies with state and federal regulations (NRC 1996). While researchers should determine which permits are needed, according to OLAW, it is the IACUC's responsibility to ensure compliance with "pertinent state, national and international wildlife regulations" (OLAW/OER/NIH 2006). Hence, the IACUC may request verification that the researcher has acquired the necessary permits before approving the collection of amphibians.

The effects of removing amphibians from the habitat must be considered. The number removed should always be the minimum required to accomplish the research goals. The total population size as well as the collection's impact on the population should be considered. The collection of animals from a breeding or hibernating/estivating aggregation or of gravid individuals can especially affect the population. In *Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research*, it is recommended that scientific justification be required when a large number of such animals are permanently removed from a population (ASIH/HL/SSAR 2004). If the collected species is the main food source for another free-ranging species, the total population should be considered to ensure that adequate food supplies are maintained for the predator species. The capture method used should also minimize destruction of the habitat as well as damage to other species (ASIH/HL/SSAR 2004).

Amphibians caught in the field are sometimes transported to a housing facility. Researchers need to ensure that the method of transport protects the individual animals from trauma, extremes in temperature or humidity, and intraspecies aggression.

If field work involves repeated capture events, or if captive animals cannot be housed individually, marking may be required to identify individuals. Methods of marking amphibians include dye marking, chemical branding, tattooing, and electrocautery/branding of the skin; microchip/passive integrated transponders or fluorescent elastomers injected subcutaneously; bands, tags, or beads attached externally; and toe clipping (ASIH/HL/SSAR 2004). The least invasive method of identifying individual amphibians is to create reference drawings of natural skin patterns (Halliday 1999).

An IACUC may interpret toe clipping either as a minor procedure (similar to a mouse tail clip) or as a surgery (an amputation), and the requirements for the method may vary. The herpetology guidelines promote caution when toe clipping. "Toe clipping should be used only for general marking of free ranging animals when toe removal is not judged (by observation of captive or of a closely-related species) to impair the normal activities of the marked animal" (ASIH/HL/SSAR 2004). Removal of more than two nonadjacent toes per foot or toes that are required for specific activities such as burrowing or amplexus is discouraged (ASIH/HL/SSAR 2004). The impact on the amphibian is unclear, but a recent study suggests that there is a negative effect. Analysis by McCarthy and Parris (2004) found that toe clipping reduces the return/recapture rate of frogs by 4 to 11% for each toe removed after the first. In addition, some species are capable of regenerating amputated tissue such as toes (NRC 1974), therefore toe clipping should be appropriate for the species. The IACUC, guided by the attending veterinarian's recommendations, should address the technique, the use of asepsis, and the need for anesthetics and/or analgesics if toe clipping is to be approved.

Amphibians used in population or habitat use studies may require tracking of individuals. If tracking is required, the IACUC must assess the methods. The use of radiotelemetry is common for many species and may be used in amphibians. Size is a limiting factor, and the IACUC should assess the transmitters weight compared with the animal—whether the transmitter would impede any activity or behavior and whether its use would interfere with the animal's continued growth. Transmitters may be attached externally, implanted in the coelomic cavity, or force fed (ASIH/HL/SSAR 2004). The appropriateness of any method should depend on the species and circumstances, and the IACUC should address the technique and use of anesthetics and/or analgesics.

Whether the animals are housed in a facility or held briefly in the field, researchers frequently perform procedures on them. In all of these cases, the IACUC must assess the method and suitability of anesthesia and/or analgesia for the procedure and the location of proposed procedures. Procedures performed on amphibians may require uncommon methods due to physiological and anatomical differences. Handling, weighing, and measuring are complicated by the skin's fragility and protective mucus layer. If animals are to be anesthetized in the field, the researcher should describe how they will be recovered and released.

Monitoring the depth of anesthesia is more challenging with amphibians than with other animals. Body temperature variations can be difficult to interpret; mucus membrane color, heart rate, and reflexes are difficult, or impossible, to observe; and respiration can be through the skin as well as thoracic. The IACUC, in consultation with the attending veterinarian, may need to discuss anesthetic monitoring with the researchers to establish an appropriate method for the specific procedure.

All animal use regulations require the use of aseptic technique for invasive, nonterminal procedures, but according to the *Guide*, allowances may be made for certain situations. The standard techniques may be modified ". . . for instance, in rodent or field surgery . . ." (NRC 1996, p. 61). The *Guide* discusses characteristics of surgery on rodents that lead to less stringent requirements such as ". . . smaller incision sites, fewer personnel in the surgical team, manipulation of multiple animals at one sitting, and briefer procedures . . ." (NRC 1996, p. 63), which are also true for many amphibian surgical situations. Appropriate disinfection of the incision site must be carefully assessed in amphibians because the skin is extremely susceptible to absorption of potentially toxic disinfectants. Chlorhexidine and benzalkonium chloride are recommended options (ASIH/HL/SSAR 2004). The attending veterinarian can determine the best practice for each species by balancing the advantages of disinfectants and the necessity of protecting the skin's defensive mucus layer. However, it is also stated in the *Guide* that "In the event of modification, assessment of outcomes should be even more intense and might have to incorporate criteria other than obvious clinical morbidity and mortality" (NRC 1996, p. 61). The IACUC may have opportunities for postapproval assessment of modified invasive procedures. The IACUC may ask the researcher for updated listings of complications, morbidity, and mortality once the procedures have begun. If animals are held in captivity, an IACUC subcommittee could visit the facility or field site to observe postsurgical animals. Animals that are available at the end of a study could be submitted for pathological examination to assess the occurrence of postoperative infection or other complications.

The IACUC must assess the method of euthanasia, which should be consistent with the guidelines of the AVMA Panel on Euthanasia (AVMA 2000). The most common method is to place the animals into a solution of buffered tricaine methanesulfonate (MS-222[1]). The solution can also be given by injection (see below, under Procedures). If MS-222 is not used, determining an appropriate method to euthanize amphibians can be complicated by their physiology and, frequently, their location. Field studies may render inhalant methods impractical and controlled drugs difficult to secure. Many species are capable of holding their breath for long periods and are resistant to cerebral hypoxia (AVMA 2000). Thus, although CO_2 is one of the safest and most commonly used methods for rodents, it is an untenable method for euthanizing amphibians. Decapitation, which is also complicated by the tolerance of amphibians to hypoxia and hypotension, requires rapid pithing of the brain. Animals intended for museum collections may require specific methods of fixation and euthanasia. For example, for some types of specimens, it may be necessary for researchers to place animals directly into alcohol or formalin without anesthesia. This method is unacceptable and would require scientific justification according to the 2000 AVMA Panel on Euthanasia and the *Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research*. According to these guidelines, only this method preserves some morphological details (ASIH/HL/SSAR 2004).

The IACUC needs to consider the proposed final disposition of captured free-ranging animals, and the protocol should state whether they will be euthanized, transferred, or released. Many amphibians are captured, restrained, measured, and released. If a procedure is performed that requires anesthesia, the method of protecting the animal until it is alert enough to protect itself is critical. If animals held captive for any period of time are to be released, the conditions must be specified. Will it be returned to the same site, or is a different site more appropriate? Is there a concern with a seasonal change during the time from capture to release? The possibility of introducing pathogens into the environment must be considered, especially for animals held long enough to become infected during captivity. It is important to consider whether the animal is to be released into a site different from the capture location or whether the animal is exposed to conspecifics from varied locations. The IACUC may determine that animals should not be returned to the field if they have been housed with other individuals or species or possibly exposed to infectious agents. In addition, local, state, or federal laws may prohibit the release of animals in some circumstances, and researchers should be asked to determine whether any of these regulations apply to their study.

Potential Zoonoses and Other Issues of Occupational Health and Safety Significance

Institutions that maintain laboratory animals for research or instructional programs require trained personnel to work with and care for the animals (Medina and Anderson 2007). Such work often poses certain hazards, which must be recognized and minimized by the institution. The Occupational Safety and Health Act requires employers to provide a safe and healthy workplace for their employees (Wald and Stave 2003). Furthermore, the NRC *Guide* and the PHS Policy require that an occupational health and safety program be a part of the overall animal care and use program. As a consequence, all individuals who are in contact with any laboratory animal species must be enrolled in an occupational health and safety program. This program must be designed to ensure that risks associated with experimental animal use are reduced to an acceptable level (NRC 1996). In the NRC *Guide* (1996), it is specifically stated that "potential hazards, such as animal bites, chemical cleaning agents, allergens, and zoonoses that are inherent or intrinsic to animal health should be identified and evaluated" (NRC 1996, p. 14) The role of the IACUC then should be to ensure that an effective occupational health and safety program is in place; that personnel working with animals are consistently enrolled in the program; and that there is effective communication between personnel, the institutional unit responsible for health and safety, and the occupational health professional to address exposure to potentially zoonotic agents.

A detailed review of occupational health and safety programs in laboratory animal research environments was presented in a previous issue of *ILAR Journal* (Wald and Stave 2003). Thus, we limit the focus of this section to key areas that include selected zoonoses and other factors that are relevant to amphibian use in research and teaching.

Salmonellosis

Salmonellosis is one of the most common fecal-oral diseases of both humans and animals, and *Salmonella* spp. are among the most recognized zoonotic pathogens (Mermin et al. 2004; Sanchez et al. 2002). Salmonellae are often thought of as a pathogen acquired through poultry or livestock, yet ectothermic pets such as reptiles and amphibians are known to harbor this bacterium (Woodward et al. 1997). Indeed, Taylor et al. (2001) indicate that "the presumption should be made that amphibians carry one or more *Salmonella* spp. at some point" (p. 18). The list of *Salmonella* spp. potentially harbored by various amphibians is beyond the scope of this article.

Amphibians may carry more than one *Salmonella* spp. and typically shed the organisms via the fecal route, therefore an aquatic environment will facilitate environmental contamination and transmission (O'Rourke and Schultz 2002). Some authors indicate that salmonellosis in amphibians may be accompanied by nonspecific clinical signs of septicemia such as anemia, lethargy, anorexia, or diarrhea (Crawshaw 1992a; O'Rourke and Schultz 2002; Raphael 1993); however, it is also likely that the organism will remain clinically unrecognized in amphibian hosts due to an absence of clinical signs (Pfleger et al. 2003; Taylor et al. 2001). Clinical signs of septicemia in amphibians and findings of enteritis at necropsy must be differentiated from other potential amphibian pathogens.

Zoonotic salmonellae cause an acute gastroenteritis in humans typified by a 6- to 72-hour incubation period after ingestion of the agent. Clinical signs of salmonellosis in humans typically consist of abdominal pain, diarrhea, nausea, and fever, with recovery in 2 to 4 days (Acha and Szyfres 2003; Fox et al. 2002). A postrecovery carrier status is possible. Laboratory, field, or animal care personnel who develop diarrheal disease concurrent with a necropsy diagnosis of amphibian salmonellosis should seek medical attention and should consider prompt examination by the institution's occupational health professional.

Mycobacteriosis

Mycobacterial infections in amphibians associated with nontubercular species are not uncommon and must be recognized as a potential zoonotic pathogen among laboratory and animal care staff who work frequently with amphibia. The most common of these pathogens is *Mycobacterium marinum*, known as the agent responsible for "fish handler's disease" or "swimmer's granuloma" (Cannon et al. 2006). Other mycobacterial agents that have been identified in amphibians include *Mycobacterium xenopi, Mycobacterium fortuitum, Mycobacterium chelonae*, and a "*Mycobacterium ulcerans*-like" agent (Acha and Szyfres 2003; Green et al. 2000; Raphael 1993; Taylor et al. 2001; Trott et al. 2004).

Mycobacteriosis in amphibians may remain subclinical or may present as cutaneous or subcutaneous nodules. Mycobacteriosis may also cause a systemic disease that is evidenced by lethargy, weight loss, and a loss of body condition. Necropsy findings are typified by multiple gray-to-white nodules of varying sizes in the liver, spleen, and other internal organs. Solitary massive granulomas in multiple organs may also be noted (Taylor et al. 2001). Occasionally, coelomic effusion may be noted at necropsy (Tarigo et al. 2006). Cytological evaluation of the lesions may demonstrate acid-fast bacilli, a valuable diagnostic tool; however, not all mycobacteria will stain acid-fast. Thus, we recommend that a diagnostic laboratory that is capable of identifying the agent through culture and polymerase chain reaction-based testing evaluate samples from suspicious animals.

Human infections from nontubercular *Mycobacterium* spp. are uncommon in North America and often present as post-traumatic wound infections on the extremities. Granulomatous skin lesions are most frequent and, in the case of *M. marinum*, may require 2 to 4 weeks to develop. Such lesions are typically acquired following wound exposure to contaminated soil or water or from handling infected animals. This clinical progression is typical of infection by most amphibian mycobacterial pathogens; however, at least one other *Mycobacterium* spp.—*M. ulcerans*—is of notable concern because it is the agent of the disfiguring "Buruli ulcer" in Africa and "Bairnsdale ulcer" in Australia (Acha and Szyfres 2003). Recent literature reports of *M. ulcerans* in colonies of *Xenopus* spp. frogs underscore this concern (Mve-Obiang et al. 2005; Trott et al. 2004). Laboratory or animal care personnel who develop ulcerative lesions on the extremities, particularly if a mycobacterial agent has been identified in an amphibian colony, should seek medical attention and should consider prompt examination by the institution's occupational health physician. Personnel who are immunosuppressed for any reason should avoid contact with amphibians potentially infected with mycobacterial species.

Chlamydiosis

Although birds are frequently implicated as a source of chlamydial infections for humans, infections of amphibian species have been well documented in the scientific literature (Berger et al. 1999; Bodetti et al. 2002; Corsaro and Venditti 2004; Hankenson et al. 2003; Howerth 1984; Newcomer et al. 1982; Reed et al. 2000; Wilcke et al. 1983). The most common or likely chlamydial agents to affect laboratory amphibia have been documented in several outbreaks; the agents appear to be *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*) and *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) (Corsaro and Venditti 2004; Crawshaw 1992b; O'Rourke and Schultz 2002). In contrast, the most common human chlamydial agent, *Chlamydia trachomatis*, is not naturally transmissible from animals (Fox et al. 2002).

Chlamydial infections are often transmitted via the respiratory route or by direct contact (Acha and Szyfres 2003); however, in amphibians, particularly *Xenopus* spp., the agent may also be transmitted via the fecal-oral route (O'Rourke and Schultz 2002). Clinical signs in amphibia are nonspecific and may vary with the species of bacterium and the host to include lethargy, loss of equilibrium, and skin sloughing (Crawshaw 1992a). Gross lesions at necropsy include pyogranulomatous lesions in liver, spleen, heart, and other internal organs. Clinical signs in humans range from a mild febrile respiratory illness to a more insidious onset of 7 to 10 days with a potentially radiographically evident bronchopneumonia. Respiratory complaints are most common, although myocarditis, hepatitis, and encephalitis have been reported (Acha and Szyfres 2003; Fox et al. 2002). The role, if any, that amphibians may play in the transmission of emerging parachlamydiacea remains to be determined (Greub and Raoult 2002). As with any other potential zoonotic agent, exposed personnel should seek medical attention and notify their institution's occupational health care provider.

Aeromoniasis

Aeromonas hydrophila, which is a common opportunistic pathogen of many amphibian species, is frequently implicated as one of the causative agents of "red-leg syndrome," the clinical description of septicemia in anurans (O'Rourke and Schultz 2002; Taylor et al. 2001). Aeromoniasis in laboratory amphibians may also be associated with exacerbating stressors such as poor water quality or pre-existing disease. Aeromoniasis in humans is typically a self-limiting enteric disease in young children, acquired through ingestion of contaminated fresh water from rivers, ponds, and lakes. An extraintestinal clinical disease syndrome has also been described in which affected humans develop cellulitis as a result of traumatic wound infection (Acha and Szyfres 2003). Despite the relatively common occurrence of aeromoniasis in laboratory amphibian colonies and despite the clear recognition of *A. hydrophila* as a human pathogen, there are only a few published reports of direct transmission of *Aeromonas* spp. from animals to humans (Acha and Szyfres 2003; Davis et al. 1978; Nemetz and Shotts 1993). Nevertheless, personnel working with amphibians should take appropriate precautions to reduce the opportunity for *Aeromonas*-related infection.

Other Potential Zoonotic Agents

Potentially zoonotic Enterobacteriaceae that have been isolated from healthy amphibians include *Edwardsiella tarda* and *Yersinia enterocolitica* (Taylor et al. 2001). The literature provides little evidence that either agent causes clinical disease in amphibians. However, identification of these potential zoonoses should be regarded with caution, and personnel should be appropriately informed to take steps to prevent ingestion or oral contact with contaminated materials.

A few zoonotic or possibly zoonotic parasitic agents are worth a brief mention. The pseudophyllidean cestodes of the tapeworm genus *Spirometra* cause visceral larval migrations and encyst to cause a disease known as "sparganosis." Humans become infected most often by consuming undercooked contaminated amphibians or reptiles, which is admittedly unlikely to occur in a research setting. Humans are less often infected through skin penetration after contact with infected animals or by ingesting drinking water contaminated with *Spirometra*-infected copepods (Acha and Szyfres 2003). Other possibly zoonotic parasitic agents occasionally identified in amphibia include *Giardia agilis* and *Cryptosporidium* species, protozoal agents known to cause enteric disease in humans and other mammals (Acha and Szyfres 2003; Green et al. 2003; Hankenson et al. 2003; Poynton and Whitaker 2002). Although there is less information in the literature describing these potential zoonotic agents in amphibians, it is nonetheless important to cautiously identify these agents in a colony and to take appropriate personal protective measures.

Commonsense Protective Measures for Personnel

Amphibians in laboratory settings or as field specimens can serve as a source for exposure to the few zoonotic agents described above despite a paucity of published information documenting laboratory or field amphibian-acquired zoonotic infections. For this reason, we advise following the methods listed below to reduce the health risk of possible exposures. The following three approaches for personal protection are the most logical and straightforward:

1. Wearing waterproof gloves of either nitrile or latex (powder free to avoid amphibian skin irritation) when handling amphibians, their tissues, or their intestinal contents;
2. Washing hands frequently and thoroughly to avoid oral contact with amphibian "products"; and
3. Wearing a protective mask and eye protection if splash hazards are present or when cleaning animal quarters.

Handwashing should immediately follow the removal of one's gloves. Likewise, one should not eat, drink, or smoke while working with amphibians (or any other research animal). Personnel should also wear laboratory coats or jackets because this practice is standard in any animal laboratory setting. Immunocompromised persons face increased risks, given that it is nearly impossible or impractical to be assured that amphibia do not carry subclinical *Salmonella* spp. Such persons should be advised by an occupational health professional whether it is appropriate to continue work with any animal species. For colonies known to be infected with mycobacterial or chlamydial agents, all personnel should consider additional precautions such as wearing ocular and/or respiratory protection and ensuring that broken skin does not come in contact with animals, their water, or associated equipment. Staff should consider colony depopulation and decontamination measures as well. Finally, we highly recommend consultation with the institution's occupational health professional. Recent publications from the Centers for Disease Control and Prevention (Bender et al. 2005; Reporter et al. 2003) and from the Association of Reptilian and Amphibian Veterinarians (Bradley et al. 1998) provide relevant published recommendations for the general public as well.

Other Occupational Health and Safety Issues

Institutions that maintain laboratory animals for research and instruction must confront a number of additional occupational health and safety issues beyond zoonosis control. Many of the same issues associated with other laboratory animals can also be applied to laboratory amphibians. The major components of an occupational health and safety program are described in the NRC publication *Occupational Health and Safety in the Care and Use of Research Animals* (NRC 1997) and its update in the *ILAR Journal* article by Wald and Stave (2003).

Many occupational health and safety issues are often identified and evaluated during IACUC inspections. Examples of IACUC responsibilities related to potential physical hazards IACUCs include the following:

- Ensuring that electrical outlets and appliances are equipped with ground-fault interrupters;
- Evaluating the integrity and appropriateness of electrical cords in a potentially wet environment;
- Ensuring that there is not an excessive amount of water on a floor, to prevent slip hazards;
- Evaluating the condition of the flooring and the placement of laboratory equipment and supplies to reduce tripping hazards; and
- Evaluating support structures of tanks or aquaria to ensure that they are stable and will not collapse, particularly if they are maintained on elevated shelving.

Certain species of amphibia may themselves pose potential hazards from the glands of the skin, such as the toxins released from the parotoid glands of the marine toads (*Bufo marinus*) or dendrobatid frogs (DeNardo 1995; St. Claire et al. 2005). The researchers and the IACUC must consider the handling, housing transport, euthanasia, and disposal of these unique animals.

The issues described above merely skim the surface of what is required for a complete institutional program. However, these matters do represent an important consideration for IACUCs during review of protocols and routine inspections.

Indicators of Stress and Disease

Because amphibians are vertebrates, their use for research, teaching, and testing is regulated by the PHS Policy. The following documents all require that pain and distress are minimized to the extent possible: the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (IRAC 1985); the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002); and the *Guide for the Care and Use of Laboratory Animals* (NRC 1996). The U.S. Government Principle IV begins, "Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative" (IRAC 1985, p. 4). Principle VII states, "The living conditions of animals should be appropriate for their species and contribute to their health and comfort . . . veterinary care shall be provided as indicated" (IRAC 1985, p. 5). Thus, pain and distress due to experimental conditions, husbandry, or disease must be addressed. To minimize pain and distress, whatever the cause, it is necessary to recognize pain and distress. Scientific investigation has provided a functional amount of knowledge about recognition of pain and distress in mammals; however, we have been unable to identify any published scientific studies investigating general signs of pain or distress in amphibians.

Amphibian behavior, or its apparent lack, also makes it challenging to interpret these animals. For the most part, they lack facial expressions and show less spontaneous movement than most mammals. In addition, many have minimal, if any, vocalization. The behavior they do display is also less well understood than mammalian behavior and can be misinterpreted. For example, pain or distress could result in decreased or increased activity. Bradley (2001) provides an empirical list of amphibian behavior responses to pain, including color change, rapid respiration, and immobility/lethargy/closed eyes. There are some behaviors that indicate specific pain, such as the "wiping response," which a frog demonstrates in the acetic acid test (Pezalla 1983). When a drop of noxious stimulus is applied to its skin, a frog will wipe the affected area with one or both hindlegs. This response is very useful for testing irritancy of solutions, but it cannot be used to assess all types of pain. Thus, determining how best to minimize pain and distress for amphibians can be challenging for researchers and IACUCs. However, there are ways an IACUC can successfully ensure humane care and use of amphibians, as discussed below.

Procedures

The first step in ensuring the humane care and use of amphibians is to ask questions about procedures that have the potential to cause pain and/or distress. Anesthesia and analgesia should be considered for any invasive procedures. The use of most anesthetics and analgesics has not been well studied in amphibians, making the choices limited and sometimes controversial. However, resources exist such as *Anesthesia and Analgesia in Laboratory Animals*, which includes a helpful section on amphibians (Schaeffer 1997). The attending veterinarian can guide the IACUC in evaluating appropriate analgesia and anesthesia. In addition, Green (2003), Machin (2001), and Wright (2001) have reviewed some agents that have been shown to have analgesic properties in amphibians.

Hypothermia has been discussed as a method of anesthesia, but its efficacy is unclear. In the Martin chapter titled "Evaluation of Hypothermia for Anesthesia in Reptiles and Amphibians," the author discusses the limited research and concludes, "Although the available articles related to the subject are inadequate for such a large and diverse group, they generally do not support hypothermia as a clinically efficacious method of anesthesia" (Martin 1995). The efficacy remains controversial, and IACUCs working with researchers who advocate hypothermia for their anesthesia must negotiate with limited scientific knowledge.

Methods for inducing analgesia with hypothermia or with immobilization stress have been investigated, and some scientific support for these methods exists (Stevens et al. 1995; Suckow et al. 1999). Clinical application of these techniques for postprocedural analgesia should be undertaken from the perspective of the relative lack of scientific data and subsequent uncertainty of efficacy. Some authors specifically discourage the use of hypothermia as a postprocedural analgesic (Green 2003; Machin 2001).

MS-222 is commonly used as an anesthetic, and it works well in aquatic species such as *Xenopus* spp. and axolotls. Terrestrial species can be placed in a few millimeters depth of buffered MS-222 so that it is absorbed through the skin. Drug doses are less well substantiated or accessible than those for mammals. The research situation itself may complicate choices (e.g., the field study location may impede the safe use of inhalants). The IACUC should seek guidance from the attending veterinarian and other consultants with relevant expertise when assessing anesthesia and analgesia to minimize pain and distress in amphibians.

One of the common uses of amphibians is collection of oocytes from *Xenopus* spp. frogs by cellular biologists. To procure oocytes at the appropriate stage, it is sometimes necessary to remove them surgically. Because performing multiple major survival surgeries on an individual animal contradicts the recommendations set forth in the *Guide* (NRC1996), it is necessary to justify more than one oocyte collection surgery. IACUCs have had to determine, somewhat arbitrarily, how many repeated intraceolomic surgeries on an individual frog are humane. The goal is to minimize pain and distress, but it is challenging to decide at what point, if ever, frogs experience more than minor or brief pain and distress. Methods of alleviating potential pain after surgical oocyte collection also need to be considered. Green (2003) discusses the possible use of postoperative analgesia, but cites the disadvantages involved and emphasizes the need for clinical studies to determine safe and efficacious drugs.

Disease

IACUCs must address the animal welfare implications of research that induces illness, such as infectious disease or tumor studies. Illness-inducing studies using amphibians require the same scrutiny as similar studies using other species. It is important to consider issues such as possible clinical disease, frequency of observing the animals, and pre-established endpoints.

Evidence of morbidity and endpoints can be difficult to establish for amphibians because common clinical signs are general and limited compared with other species. However, there are several common clinical signs that may be observed in amphibians, which include skin lesions (hyperemia, discoloration, ulceration, and texture changes), fluid retention (ascites or anasarca), cloacal prolapse, weight loss, weakness, anorexia, lethargy, tumors, and loss of righting reflexes. When signs are noted, it may be difficult in some cases to determine the severity and know whether an endpoint has been reached. Lethargy and weakness are especially challenging to categorize for an animal that normally moves very little. Anorexia can also be challenging to establish in species that normally do not eat for days at a time. Handling an amphibian to examine its reflexes or monitor its weight may endanger its health because damage to the skin or its mucus layer provides access for infectious agents.

The IACUC must also ensure that the care of amphibians in captivity minimizes stress and the risks of spontaneous disease. Preventive care may include quarantine and separation of species or groups with different health statuses. Amphibians are very sensitive to environmental parameters, and minor environmental problems can cause distress, severe illness, or death. Environmentally stressed amphibians are susceptible to clinical disease from common subclinical agents such as *A. hydrophila* or *Mycobacterium* spp. Daily monitoring of animals for indications of disease should be performed as for any laboratory animal.

Also for amphibians, it is necessary to control for the parameters of concern for mammals. Temperature, humidity, lighting levels and cycles, noise, population density, nutritional requirements, and frequency of feeding affect the animals' health. While enrichment with substrates from the natural environment is frequently attempted to minimize stress, it is important to choose an appropriate substrate and to consider its potential to harbor infectious agents.

Water quality is vitally important for amphibian health, especially for aquatic species. Water parameters that can affect the health of all amphibians include pH, temperature, alkalinity, and hardness (Godfrey and Sanders 2004). For aquatic species, the organic byproducts ammonia, nitrate, and nitrite must be controlled with biological filtration. Different species tolerate variable levels of turbidity and turbulence. The levels of dissolved gases (oxygen, carbon dioxide, nitrogen, and hydrogen sulfide) are important, and aquatic environments should not contain contaminants such as heavy metals, chlorine and chloramine, or other chemicals (Cassidy 2006). Water temperature (Green 2002) and hardness (Godfrey and Sanders 2004) also affect *X. laevis* oocyte quality.

In summary, the IACUC has a critically important role with regard to monitoring the environment of amphibians used in research. The IACUC must ensure that all of the environmental parameters for the specific species have been determined with the best available resources, are able to be controlled, and are adequately monitored.

Conclusion: IACUC Resources

Many IACUCs will eventually have to address the use of amphibians for research, teaching, and testing. It can be daunting for members with little previous exposure to amphibian-related issues. The federal animal welfare regulations offer the IACUC some assistance. The AWA does not cover amphibians, but the PHS Policy and the NRC *Guide* do include amphibians and do provide some guidance. Although the NRC *Guide* contains few amphibian-specific recommendations, many of the general recommendations apply. We also direct readers to the additional amphibian resources that appear in the appendix at the end of this article.

It is important for IACUC members to realize that resources exist and should be used to make informed decisions. Frequently, the researchers can provide information about the specific species, and especially information about proper husbandry. The IACUC should also consider requesting advice from laboratory animal veterinarians, scientists, or others with amphibian expertise. Potential consultants are researchers who use amphibians at the IACUC institution or other institutions, managers of zoological or other live amphibian collections, members of state or federal natural resources departments, and veterinarians with experience caring for amphibians. Additionally, written documents are available that have been accepted by the laboratory animal industry as providing general care and use standards.

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Appendix: Resources for the Care and Use of Amphibians

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