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Canadian Council on Animal Care
Conseil canadien de protection des animaux

CCAC guidelines: Amphibians

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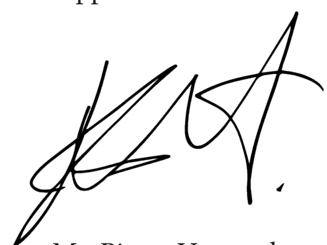

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The Canadian Council on Animal Care (CCAC) is the national peer-review organization responsible for setting, maintaining, and overseeing the implementation of high standards of ethical care and use of animals in science throughout Canada.

The *CCAC guidelines: Amphibians* provides information for investigators, study directors, animal care committees, facility managers, veterinarians, and animal care staff to help facilitate improvement in both the care given to amphibians and the manner in which experimental procedures are performed. These guidelines address conditions normally present in laboratories housing amphibians; where experimental conditions required by studies differ from the guidelines, they must be justified to and approved by the animal care committee.

CCAC guidelines are intended to provide assistance in the implementation of Russell and Burch's Three Rs (Replacement, Reduction, and Refinement) principles for animals in science (Russell and Burch, 1959). The guidelines are based on expert interpretation of current scientific evidence and have been subject to peer review. They are intended to provide a framework for the implementation of evidence-based practices, which are constantly evolving. Implementation of evidence-based practices should result in continual improvement in animal welfare.

For studies outside of Canada, investigators based at CCAC-certified institutions are subject to these guidelines and the relevant legislation and regulations pertaining to ethical animal care and use in the country where the study is conducted.

SUMMARY OF THE GUIDELINES LISTED IN THIS DOCUMENT

The following list of guideline statements serves as an executive summary covering the most important aspects of the care and use of amphibians. These guideline statements are included throughout this document alongside details and references that provide support and context for their implementation. Throughout this document, the term 'must' is used for mandatory requirements. The term 'should' is used to indicate an obligation, for which any exceptions must be justified to and approved by an animal care committee.

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1 INTRODUCTION

Throughout this document, the term 'must' is used for mandatory requirements. The term 'should' is used to indicate an obligation, for which any exceptions must be justified to and approved by an animal care committee.

Amphibians are a class of ectothermic (cold-blooded) vertebrates, comprised of three families: Anura (frogs and toads), Caudata (salamanders, newts, etc.), and Gymnophiona (caecilians). They undergo metamorphosis, which is generally characterized by an aquatic larval stage, followed by a semi-terrestrial adult stage. However, some species within this group have direct development (e.g., some plethodontid salamanders), and others, such as *Ambystoma mexicanum*, remain fully aquatic for their entire lives.

The various species of amphibians display a wide range of adaptations and have a diversity of environmental requirements (Elinson and del Pino, 2012). In addition, amphibians are profoundly unlike mammals. They require a functioning microbial community in their immediate surroundings to maintain an adequate skin microbiome to ward off disease. Therefore, it is critical to be aware of the individual needs of the animals and how their welfare is influenced by their housing environment and any studies they are involved in.

The *CCAC guidelines: Amphibians* focuses on amphibians held in laboratory facilities or brought into facilities from the wild. For studies involving amphibians in the wild, including short-term holding[1] in the field, see the *CCAC guidelines on: the care and use of wildlife* (CCAC, 2003b). Amphibian eggs, embryos, or larvae that have not developed beyond exclusive reliance on their own yolk nutrients (i.e., have not begun external feeding) are not covered by these guidelines[2], as the CCAC does not require an animal use protocol for these life stages.

These guidelines are intended to be applied to all amphibian species held in Canadian facilities (see Appendix 1 for a list of commonly held species); however, greater detail is provided for those species that are most often housed (*Xenopus laevis* (African clawed frog)[3] and *Ambystoma mexicanum* (axolotl)) where it is available. Because of the diversity within this group of vertebrates, these guidelines should be used as general standards for their care and use in science. It is critical that those working with a particular species have a comprehensive understanding of the housing and husbandry requirements of those animals, which can be acquired through literature searches and consultation with investigators, veterinarians, and others who have experience with that species. Where knowledge of optimal conditions for a species is lacking, these guidelines and the species' natural habitat provide an appropriate starting point, which should be followed up with careful monitoring and adjustment.

1 Short-term holding is defined by the American Society of Ichthyologists and Herpetologists (2004) as days to weeks.
2 For example, in anurans, the guidelines apply at Gosner stage 24; see McDiarmid R. and Altig R. (2000) *Tadpoles: The Biology of Anuran Larvae*. University of Chicago Press.
3 These frogs are commonly referred to by their scientific name, *Xenopus*, which is used throughout this document.

Amphibians have been used as models for developmental and physiological processes, including tissue regeneration studies, and more recently, genetic and genomic research (O'Rourke, 2007; Edholm and Robert, 2013). Amphibians also play an important role in environmental research related to environmental contamination and climate and habitat changes (O'Rourke, 2007).

Some of the challenges associated with amphibian-based studies include:

- different housing and care requirements at each developmental stage;
- lack of knowledge of amphibian welfare;
- limitations in recognition, evaluation, and alleviation of pain, discomfort, and distress;
- limited information on behavioural testing;
- technical difficulties inherent in studies involving injections, blood collection, and surgical procedures due to the small size of some amphibians;
- difficulty in maintenance of aseptic technique for recovery surgery;
- potential negative effects on animal welfare of transgenic models and other specialized disease models being generated;
- lack of availability of some species through suppliers;
- capture from ecosystems where amphibian species are threatened; and
- concerns regarding the release of captive wild amphibians that have been held in facilities.

As with all animal-based science, the scientific validity of any protocol involving amphibians must be established carefully, and the Three Rs (Replacement, Reduction, and Refinement) (Russell and Burch, 1959) must guide decisions concerning experimental design and the care of the animals. Consideration of the quality, reproducibility, and translatability of studies is critical throughout the design, conduct, and reporting of studies to ensure that anytime an animal is used in science, their life is not taken in vain and the data obtained contributes to the research record.

Replacement is an important consideration in planning animal-based studies. Consideration must also be given to reduction to determine the smallest number of animals consonant with the provision of valid information and adequate statistical power, while minimizing the welfare impact for each animal. Sample size calculations should be carried out, and when necessary, a biostatistician should be consulted.

The present guidelines focus primarily on refinement, both in terms of the care of amphibians in a facility and the procedures carried out on amphibians as part of an animal-based protocol that requires approval from an animal care committee. Animals living in an environment where facilities and practices are oriented toward promoting good animal welfare are less likely to be stressed and more likely to exhibit normal behaviours and physiology (Poole, 1997).

The following sections provide a brief overview of the behavioural biology important to the welfare of amphibians (Section 1.1, "Behavioural Biology"), the sensory abilities of amphibians (Section 1.2, "Senses"), the particular anatomical and physiological characteristics of amphibians (Section 1.3, "Anatomy and Physiology"), and potential inter-animal variations (Section 1.4, "Sources of Variation"), which have an impact on welfare considerations and form the basis of this guidelines document. It is important to incorporate knowledge of the characteristics of the species (and strain where applicable), sex, developmental stage, and individual animals when considering the impact of a procedure or condition on the welfare of amphibians and on the research results.

See Appendix 1 for potential resources to consult for additional background information on particular species.

1.1 BEHAVIOURAL BIOLOGY

Addressing the welfare of amphibians in the laboratory requires consideration of their natural behaviours (which vary with species) and providing the opportunity for those behaviours to be expressed where appropriate. In general, the life history of amphibians includes an aquatic larval stage, followed by metamorphosis to the adult form; however, there is a great deal of variation in life cycle among species (Pough, 2007). The adult form may be fully aquatic, semiaquatic, or fully terrestrial, depending on the species; this range occurs within both *Anura* (frogs and toads) and *Caudata* (salamanders and newts) (Pough, 2007).

Modes of locomotion for adult amphibians include jumping, swimming, walking, running, and climbing, depending on the species (Pough, 2007). When undergoing metamorphosis from the aquatic larval form, amphibians require a landing area to rest in order to prevent drowning. However, spontaneous metamorphosis is extremely rare in axolotls, and a landing area is not necessary unless indicated through regular monitoring.

As ectotherms, amphibians regulate their body temperature through behavioural means (i.e., moving into an area with an appropriate microclimate) and evaporative cooling (Tinsley, 2010). Temperature preferences vary among species, and within a particular species, they can vary daily and seasonally (Pough, 2007). Some species enter a state of torpor at very low temperatures (Tinsley, 2010).

Juvenile and adult amphibians are carnivores (primarily preying on invertebrates); however, the diet of larval amphibians is more variable and includes a herbivorous phase for some (Tinsley, 2010).

Amphibians generally prefer to spend a large portion of the day hidden from sight (Pough, 2007), occupying shaded areas and avoiding bright light. Many amphibians are nocturnal and feed at night (Pough, 2007). Reproductive cycles and metamorphosis are also influenced by photoperiod, among other factors (Pough, 2007).

Some amphibians tend to be solitary except during breeding (Tinsley, 2010), while others prefer to be in groups; therefore, it is important to know what is optimal for the species of interest. Their social behaviour can be complex (Wells, 2007), and some amphibians use vocalizations (Wells and Schwartz, 2007; Tobias et al., 2004), displays (Hodl and Amézquita, 2001), or chemical signaling (Pough, 2007) to communicate with conspecifics regarding dominance, territory, and mate availability.

1.2 SENSES

Many amphibians use vision to detect the presence of prey. They respond to the movement of prey and may not respond to a food item that is stationary or does not resemble the size and shape of common prey species found in their natural habitat (Pough, 2007). Some amphibians, such as salamanders, use both vision and smell to detect prey, while *Xenopus* rely primarily on smell (Reed, 2005). Smell may also be involved in courtship, mating, and territoriality in various amphibian species, in response to the release of chemical signals from cutaneous glands (Woodley, 2014). Some larval and adult amphibians have a lateral line system that responds to changes in water currents and water pressure and can play a role in locating food (O'Rourke, 2007) and detecting local threats such as predators.

For some species (e.g., frogs), sound production and hearing have been extensively studied, whereas for other species (e.g., salamanders), the research in these areas is sparse. Nevertheless, research indicates that amphibians can hear high (air-borne) and low (substrate-borne) frequency sounds (Capshaw and Soares, 2016). Some species produce complex vocal signals that are used in mate attraction and territoriality. Furthermore, the structure of these calls can contain information pertaining to an individual's fitness and reproductive status.

Amphibians can hear in sound frequencies as low as 10 Hz and as high as 10 kHz (Heffner and Heffner, 2007; Capshaw and Soares, 2016). For reference, humans are able to hear as low as 20 Hz and as high as 20 kHz. Research on the impact of sound levels in both the field and laboratories suggests that loud sounds, such as those produced by traffic, can result in breeding inhibition, increased vigilance and increased levels of stress hormones (Morgan and Tromborg, 2007; Tennessen et al., 2014).

All three orders of amphibians are capable of sensing ground vibrations, and caecilians use this sense for burrowing. While laboratory studies on sound and vibration tend to focus on mammals, there is anecdotal evidence that reproduction in amphibians is disturbed by vibration. Amphibians may not exhibit an overt response when experiencing stress; for example, axolotls do not show obvious behavioural responses to vibration or changes in light and temperature; however, reproduction can be affected.

1.3 ANATOMY AND PHYSIOLOGY

It is very important to be familiar with the anatomy and physiology of the particular species being housed or used in research. Failing to recognize species-specific details may result in poor welfare of the animals and may have negative impacts on studies. Some features that are important to amphibians are stated in the paragraphs below.

Amphibians have a thin, permeable skin that allows the exchange of oxygen and carbon dioxide and the movement of water into and out of the body (Pough, 2007). Drugs and toxic substances can also enter through the skin. Amphibians excrete toxins through their skin or from specialized parotoid glands to defend against predators and disease-causing organisms.

Amphibian skin produces antimicrobial compounds, and the skin microbiome plays a vital role in fighting off bacterial and fungal infections (Walke and Belden, 2016; Jiménez and Sommer, 2017). Many species have a protective mucous layer covering the skin, and damage to this layer can cause skin lesions or infections. In addition, the captive environment of amphibians has been shown to have negative effects on their microbiome (Bates et al., 2019).

Amphibians undergo a moulting cycle, in which the outer layer of the epidermis is shed. The frequency of moulting has been linked to temperature, ambient light (Cramp et al., 2014), illness, and injury (Ohmer et al., 2015).

Most amphibians require water for laying eggs, even terrestrial species. The eggs have a protective jelly coat, which varies in thickness depending on the species and environment (Gomez-Mestre et al., 2006). The development of larvae and their metamorphosis into young adults is influenced by environmental conditions (Denver, 2019). For example, the rate of development and the size of *Rana pipiens* tadpoles are influenced by temperature (Wells, 2007).

Amphibians have a well-developed nervous system with nociceptors and pathways for the perception and processing of noxious stimuli (Guénette et al., 2013). Nociception is transmitted to the central nervous system; however, there is limited information on whether these pathways reach the brain center, resulting in conscious perception of pain (Guénette et al., 2013). Due to the limitations of the evidence, the precautionary principle should be applied such that all necessary attempts are made to minimize potential suffering.

Many amphibians have the natural ability to regenerate various tissues, such as limbs, jaw tissue (Song et al., 2010), skin, apex of the heart, spinal cord, sections of the brain, and sections of the intestine. However, salamanders (in particular axolotls and three species of newts) have been more extensively used to study tissue regeneration due to the extent of their capacity for regeneration (Joven et al., 2019). While many amphibians are capable of tissue regeneration, there are energetic costs associated with it (Maginnis, 2006). This should be a consideration for invasive procedures involving the surgical removal of tissue (e.g., clipping a toe or tail), which probably also result in nociception.

Amphibians lack a diaphragm, and therefore, drugs that are injected into the abdominal cavity can move to the heart and lungs.

1.4 SOURCES OF VARIATION

While variation among species of amphibians has been acknowledged, consideration must also be given to the variation within a species when determining housing and husbandry requirements and the effects of particular procedures on the welfare of the animals and the interpretation of study results.

1.4.1 Strain

A few species of amphibians have been subject to genetic modification (e.g., transgenics and knock-outs), for example, axolotls and *Xenopus* sp. For these species, there are multiple strains, each with distinctly defined genotypes and phenotypes. While there has been a recent increase in the generation of genetically modified amphibians, for most other species, there have not been any genetically modified strains produced to date, and only wild types exist.

1.4.2 Developmental Stage

A unique feature among many amphibians, compared to other tetrapods, is that they undergo metamorphosis from the larval to the adult stage (Tinsley, 2010). However, the progression of an animal through the life cycle stages varies among species. For information on development stages, see Gosner (1960) for Anurans; Nieuwkoop and Faber (1967) for *Xenopus*; Harrison (1969), Russell and Watson (2000), Kerney (2011), and Hurney et al. (2015) for various species of salamanders; and Armstrong and Malacinski (1989) and Nye et al. (2003) for axolotls. There is a great deal of variation in requirements (e.g., in terms of containment, environment, and diet) and responses (e.g., in relation to procedures) between life stages, and these variations differ between species.

1.4.3 Individual Differences – Sex and Health Status

The importance of recognizing and understanding the implications of sex differences in research has been well established (McCarthy et al., 2012). McCarthy et al. (2012) and Becker et al. (2005) discuss considerations for identifying the nature and implications of sex differences in studies to improve study design and interpretation of results. A typical example in amphibians is sexual size dimorphism, which for most species takes the form of larger females than males (Kupfer, 2007). For *Xenopus (Silurana) tropicalis*[4], males and females also display behavioural differences, with males showing more exploratory behaviour than females, and within each sex, individuals have been categorized as bold, intermediate or shy (Videlier et al., 2015). Seasonal variation may also be a factor.

The health status of amphibians has implications for their use in research and breeding and their housing requirements within a facility. While diseases affecting amphibians are not as well-known as those of many other types of animals housed in laboratories, quarantine, health monitoring programs, and other means of monitoring the colony for pathogens are important in maintaining animals of a particular health status (see Section 8, “Health and Disease Control”).

1.4.4 Effects of the Environment and Previous Experience

Differences in housing and husbandry conditions can result in variation between individuals of the same species. For example, oocyte quality has been shown to differ between *Xenopus* housed with recirculating water versus static water (Delpire et al., 2011), and some salamander larvae may develop into cannibalistic morphs based on environmental conditions, including conspecific density and food supply (McLean et al., 2016). Within the same type of enclosure, environmental factors that can be a source of stress and influence the animal’s behaviour and physiology include the density of animals, the presence or lack of retreat sites, an increase or decrease in water temperature or light intensity, noise, the presence or absence of a floating surface, the type of substrate, and the maximum water depth and any gradation within the enclosure. For terrestrial animals, humidity is also an important factor. Additionally, the health and development of individuals can be affected by parasite infection, and the effect of this can be further influenced by the density of the amphibians (Koprivnikar et al., 2008).

Water quality can affect the health and condition of amphibians. Aging water is important for the development of a sufficient microbial population that will allow amphibians to maintain a functional skin microbiome. Water is also aged to reduce chlorine; however, the levels may still be too high for amphibians, and another treatment is required. Chloramine is also harmful and is not reduced by aging water. Additionally, amphibians (particularly larvae) are sensitive to metals found in municipal water, such as copper leached from copper piping, which is not detected by standard water quality test kits. Other variables associated with water quality that can have an impact are dissolved oxygen, pH, and the accumulation of animal waste. For more information, see Section 3.1.3.2, “Water Quality”.

It is important to be mindful of whether individual animals are wild-caught or captive-bred, as there are differences in how they acclimate to a new situation. For instance, captive-bred individuals raised in groups are less likely to exhibit aggression in new group-housing situations; however, wild-caught individuals can initially show high levels of aggression when placed in group housing (Cikanek et al., 2014). Routine husbandry practices that include handling of individuals can also be a source of stress for amphibians. In some species, acute and repeated handling results in reduced locomotor activity (Bliley and Woodley, 2012;

4 Note: *Xenopus tropicalis* is of the subgenus Silurana, and therefore, also appears in the literature as *Silurana, tropicalis* (see NCBI Taxonomy Browser).

Wack et al., 2013), feeding, and body weight (Bliley and Woodley, 2012). In addition, wild-caught animals from different locations may vary in the length of time it takes to acclimate to the environmental conditions in the laboratory.

For species that rely on crypsis or camouflage as an anti-predator response, the colour of the environment that they are housed in can potentially affect behaviour and physiology. Though relatively few studies have assessed such effects, there is evidence that some species show preference for a background colour that either matches their own body colour or is darker (Garcia and Sih, 2003; Wente and Phillips, 2005). Furthermore, a recent study showed negative behavioural and physiological impacts in *Xenopus* when housed in a tank with a white background, which does not have ecological relevance to these animals (Holmes et al., 2016).

2 FACILITIES

For general guidance on facilities, see the *CCAC guidelines on: laboratory animal facilities – characteristics, design, and development* (CCAC, 2003a). Additional guidelines and information of particular concern for amphibians are presented in this section.

2.1 ANIMAL ROOMS AND PROCEDURE ROOMS

Rooms housing amphibians must have the following features:

- floors designed to support the weight of tanks and racks (Browne et al., 2007) and sloped for drainage;
- waterproof floors, walls, and ceilings that facilitate cleaning (Browne et al., 2007);
- materials that withstand high humidity (Browne et al., 2007);
- ground fault electrical systems, with waterproof electrical outlets that are located away from water lines, where possible, and placed above the splash zone;
- a mesh-covered floor drain to prevent animals from escaping; and
- means of restricting access to designated personnel only.

Facilities should ensure there is adequate separation between the primary containment of different species, as there is the potential for the transmission of some pathogens (Densmore and Green, 2007).

Within facilities housing amphibians, there must be a designated wet area for washing and sanitizing equipment and an area for quarantine of new or sick animals (Browne et al., 2007). Depending on the species and the purpose for which they are kept, designated areas are also needed for reproduction, larval rearing, hibernation, and aestivation (Browne et al., 2007).

Rooms should be designed to prevent escape or entrance of insects (e.g., baffles on vents and door seals).

While precautions must be in place to prevent escape from enclosures (see Section 2.2, "Animal Enclosures in the Laboratory"), for some amphibians, it is beneficial to provide a refuge on the room floor that is accessible to an amphibian in the event of an escape. This refuge should hold enough water to keep the animal safe until the next day when it can be returned to its enclosure.

Rooms should be temperature and humidity controlled, with emergency alarms. Rooms should have the capacity to maintain humidity around 50%. The requirement for a constant environment is not as stringent for amphibians as it is for some other laboratory species, and amphibians can tolerate a short period without power if it is interrupted. However, an emergency power source should be available as necessary to maintain a healthy environment for the animals. Requirements for emergency power depend on the species and type of housing and may include power for air compressors, heaters, filters, water pumps, air conditioning units, humidifiers, and light sources.

2.1.1 Water Supply

Guideline 1

Facilities must be able to provide amphibians with water of suitable quality to maintain their health.

If municipal water is used, chlorine and chloramines must be removed or bound chemically, as they are toxic to amphibians (Browne et al., 2007). Depending on the species, the water source, and the type of holding system, water may need to be oxygenated, which can be achieved through aeration (Browne et al., 2007). This process can also remove volatile compounds (Browne et al., 2007). The use of a separate reservoir (holding tank) is recommended to facilitate chlorine evaporation and to act as an emergency water source in case of disruption of the water supply. However, chloramines do not off-gas or evaporate and must be removed through the use of sodium thiosulfate or carbon-based filters. In large-scale or automatic flow-through systems, chlorine alarms should be in place.

Reverse osmosis (RO) or deionized (DI) water should be considered if the source water contains harmful levels of contaminants. Charcoal filtration is also an option. Reverse osmosis and distillation remove beneficial trace elements and produce water that is significantly lower in solute concentration than the body tissues of amphibians, which can lead to bloating and kidney problems (Odum and Zippel, 2011; Poole and Grow, 2012). Therefore, treated water (RO or DI) must be reconstituted through the addition of salts and trace elements, as described by Odum and Zippel (2011) and Poole and Grow (2012), and allowed to age to reconstitute a beneficial microbial community.

Water containing copper or lead leached from pipes and phosphates that have been added to prevent leaching is toxic to amphibians (Browne et al., 2007). Harmful compounds (bisphenol A, bisphenol S, phthalates, nonylphenol, etc.) can also leach from plastic piping (Browne et al., 2007) or other plastic products. Glass or stainless steel should be used when possible; if plastic is used, it should be high-density polyethylene (HDPE) plastic or "aged" plastic. If tap water is used, it should be run for 5-10 minutes before use to reduce the level of contaminants from the pipes.

The source water should be tested for other variables, such as salinity, alkalinity and hardness, fluorides, heavy metals, microorganisms, pH, and toxicants, to ensure they are within the range suitable for the species being held. Where information on appropriate water quality is lacking for a particular species, consideration should be given to their natural habitat. See Section 3.1.3.2, "Water Quality", for guidance on acceptable ranges of water quality variables and on-going monitoring of water quality. The water in enclosures should be tested prior to introducing amphibians.

2.2 ANIMAL ENCLOSURES IN THE LABORATORY

Guideline 2

Enclosures must provide sufficient space and complexity to enable amphibians to perform behaviours important to their welfare.

All enclosures must provide fresh water, control of lighting and temperature, and the ability to monitor and access individual animals (Browne et al., 2007). Enclosures should be safe for amphibians and personnel and be easy to maintain (Browne et al., 2007).

Each species and life stage requires specific features in their enclosures to meet their physiological and behavioural needs and prevent escape (Browne et al., 2007). When live insects are provided, enclosures must also prevent their escape (Browne et al., 2007).

Terrestrial and semi-terrestrial amphibians that are close to metamorphosis or at a later stage of the life cycle should be provided with a water dish large enough to allow them to submerge and be given the option of whether or not to be under cover. There must also be a sufficiently large landing area to enable them to be out of the water.

Fully aquatic amphibians, such as mudpuppies (*Necturus*), axolotls, larval amphibians, and some adult frogs (e.g., *Xenopus*), should be held in enclosures with dechlorinated water of suitable temperature and an appropriate air supply. Airstones may be used in some situations, but they create too much turbulence for some larvae and are difficult to clean. Glass Pasteur pipets are a good alternative to airstones and can be connected to air lines to provide less vigorous aeration.

Flow rates on filters should be set to low settings, or inflowing water should be aimed or interrupted such that larvae and aquatic amphibians can rest and move throughout their enclosures without being exposed to water turbulence. Axolotls do not do well in circulating water; it is a source of stress, and a low flow rate with a rate of water exchange of 5-10% per day should be maintained (Khattak et al., 2014). Axolotl larvae, from hatching until 4 cm, should be kept in small static containers with manual water changes to prevent injury from water flow (Khattak et al., 2014).

Aquatic amphibians should be provided options for hiding and floating that are appropriate for the species. For example, full or halves of high-density polyethylene (HDPE) plastic tubing allow hiding, while winter-fence plastic can be used for floating platforms. However, any such materials added to an amphibian's environment should have smooth edges.

Arboreal amphibians require structures for climbing and resting and a water dish that allows them to submerge themselves (Forbes et al., 2007). Structures that provide the option of visual barriers between animals and allow them to hide from people may be useful.

2.2.1 Spatial Requirements

At a minimum, amphibians must be provided with sufficient space to perform the following general behaviours; however, larger enclosures are encouraged:

- terrestrial and semi-terrestrial animals – stand, extend to their full length, turn around, soak in water (depending on the species), find cover, and rest;
- aquatic animals – swim, extend to their full length, turn around easily, access the surface and bottom of the tank, find cover, and rest/float;
- arboreal animals – stand, extend to their full length, turn around, climb, and rest.

2.2.1.1 *Xenopus*

The density of animals, floor space of the tank, and depth of the water must support the health of the animals and allow for normal behaviours. The amount of floor space in tanks should allow each frog to be on the floor without touching other frogs. Optimal conditions for *Xenopus laevis* and *Xenopus tropicalis* will differ due to their substantial differences in size.

Tinsley (2010) notes that recommendations for the density of adult *Xenopus laevis* range from 2 L per animal to 12 L per animal and suggests that 2 L per animal is too restrictive, while 12 L per animal may be impractical. Other published recommendations for *X. laevis* are in the range of 2.6-3.8 L per animal, using a recirculation system with 10% water replacement per day (Kroll Lab, 2012; University of California, Berkley, 2018; McNamara et al., 2018). Due to the smaller size of *Xenopus tropicalis*, 1 L per animal has been recommended (Kroll Lab, 2012; McNamara et al., 2018). For juvenile frogs, McNamara et al. (2018) recommend one animal per 1-2 L for *X. laevis* (8-12 months old) and one animal per L for *Xenopus tropicalis* (5-8 months old). The sex of the animals and the type and purpose of housing (e.g., breeding) should also be taken into consideration. Adult males and females should generally be housed separately.

The minimum water depth for adult *Xenopus laevis* that enables the performance of most normal behaviours is 20-30 cm (Reed, 2005; Tinsley, 2010), but a depth of 50 cm is preferable (Tinsley, 2010). There must be adequate air space between the surface of the water and the lid to prevent injury if the frogs jump.

2.2.1.2 Leopard frogs (*Lithobates (Rana) pipiens*)

When *Lithobates (Rana) pipiens* are maintained in a state of simulated winter torpor, they may be kept in fairly large numbers in large, shallow, flow-through tanks. The water level must not come above the nares to prevent drowning.

The density should be no more than 20-30 frogs per m² of floor area; however, this will be influenced by the ability of the system to remove wastes. A 1 m² tank with a water flow rate of 10-15 L per minute at 4 °C can provide suitable conditions for up to 50 frogs when necessary. Both static and flow-through systems are acceptable if environmental variables remain stable.

2.2.1.3 Larvae

The density and abundance of larvae should enable normal behaviour and good health. Their growth and development are species-specific and have been shown to be negatively affected by density; however, with appropriate housing and husbandry systems, larvae of some species can be raised at high density (Browne and Zippel, 2007). Yagi and Green (2016) note that animal abundance (separate from density) is also an important factor. For example, Fowler's Toad tadpoles benefit from the presence of a high number of tadpoles (high abundance) to promote efficient feeding and the creation of a microclimate with optimal temperature; however, each tadpole also needs sufficient space (low density) for normal growth (Yagi and Green, 2016).

Individuals showing large differences in size or growth rate should be separated unless involved in studies requiring density to be kept constant. Size variation among tadpoles can provide a competitive advantage for larger animals, and further contribute to differences in growth rates (Warne et al., 2013). In addition, cannibalism may occur in some species (e.g., larval salamanders, American toad tadpoles, and spadefoot toad tadpoles). These situations can be prevented or at least minimized by size grading or ad libitum feeding; however, if cannibalism continues to occur, the animals must be separated.

2.2.2 Enclosure Design

The shape of the enclosure should promote natural movement and social behaviour. For aquatic amphibians, long, narrow enclosures should be avoided as they limit such behaviour (Council of Europe, 2004, cited in Reed, 2005; Forbes et al., 2007).

Enclosures for semi-aquatic amphibians require landing areas and water at a depth that allows the animals to submerge (Forbes et al., 2007).

For most amphibians, enclosures should have dark sides to replicate their natural environment (Forbes et al., 2007). However, to facilitate observation of the animals while minimizing disturbance, it may be necessary to have a transparent segment of the wall (e.g., the front panel) (Reed, 2005).

Enclosures must have lids that prevent escape. Mesh lids are preferable as they allow the movement of air and light between the room and enclosure. Galvanized mesh contains high levels of zinc, which may be a problem for some species. The appearance of rust is also a concern.

2.2.2.1 *Xenopus*

Efforts should be made to house *Xenopus* in enclosures with dark sides, which resemble their preferred environment (Reed, 2005; Tinsley, 2010). Higher levels of stress, as indicated by corticosterone, behaviour, and changes in body mass, have been found in *Xenopus* housed in enclosures with a white background rather than a dark background (Holmes et al., 2016). In addition, opaque-sided enclosures are recommended as *Xenopus* are easily startled.

2.2.3 Water System

Amphibians should be provided with water through a gentle flow-through system, a recirculating system, or a static system with the water changed at least twice a week (after each feeding) to help preserve water quality (Forbes et al., 2007). For aquatic amphibians, it is important that the water is maintained at an appropriate temperature for the species and there is adequate aeration (Forbes et al., 2007). For some species, such as *Xenopus* or axolotls, it is important that any water turbulence is low and the water in the tank is kept relatively still (Tinsley, 2010) (i.e., aeration equipment that causes turbulence should not be used).

2.2.4 Substrate

The type and amount of substrate depend on the species, life stage, and type of enclosure. For example, most toads require a substrate that is deep enough for burrowing, and small salamanders (e.g., most plethodontids) do well in moist sphagnum moss.

It is important that the environment of amphibians be kept clean but not sterile to promote their health, which includes a healthy microbiome. Browne et al. (2007) suggest the following materials as substrate: artificial potting soil that is free of fertilizer, green moss, sphagnum moss, peat moss, leaf litter, gravel, ground coconut pith, and plastic grid. Consideration must be given to the potential for the substrate to be ingested and cause intestinal impaction. Fine sawdust and small-particle substrates should not be used (Forbes et al., 2007; Browne et al., 2007). Naturalistic enclosures with bioactive substrate can be difficult to maintain, particularly when large numbers of animals are being housed. Anurans and salamanders have been found to do well when provided with a moist paper substrate (Pough, 2007). However, if paper is used, it should be inert (e.g., laboratory filter paper), as other products may contain acids and bleaches that are harmful to amphibians due to their permeable skin (Pough, 2007).

2.2.5 Furnishings

Many amphibian species rely on camouflage for protection against predators. For these animals, the provision of a shelter and hiding places is important, as they will likely experience stress if not given the opportunity to avoid exposure (Pough, 2007).

While natural features such as soil and plants may be aesthetically pleasing to personnel, it is not clear whether amphibians prefer these to artificial elements (e.g., plastic pipe and plastic aquarium plants), and they are more difficult to clean (Pough, 2007). In general, materials added to enclosures should be food-grade plastic that can be cleaned easily. When artificial furnishings are used, they should not have sharp edges.

For semi-terrestrial amphibians, additions to the enclosure may include stones, pieces of artificial bark, artificial branches and leaves, and shelves (Forbes et al., 2007). Where natural materials are provided, they should be inspected regularly and sanitized or replaced as needed to minimize risks associated with physical injury and microbiological burden. If a pool of water is provided, furnishings should be placed to ensure the animals can breathe and easily access resting areas.

Salamanders require structures to enable hiding and will often be found under their water dish. Structures should be low or partially buried to enable the animals to maintain body contact with the structure walls. PVC pipe works well for this purpose.

Arboreal species require structures for climbing and resting (Forbes et al., 2007).

2.2.5.1 *Xenopus*

Xenopus are prey animals, and refuges suited to their life stage should be provided for hiding (Kreger, 2002), unless there are adverse effects on water quality. Options for refuges include commercial plastic piping used to deliver water (Brown and Nixon, 2004, cited in Reed, 2005) or plastic containers designed to store food for people (Schultz and Dawson, 2003, cited in Reed, 2005). Ceramic tiles or terra cotta pots may also provide suitable refuges, but they should first be evaluated for potential leaching of toxic agents (Reed, 2005).

3 FACILITY MANAGEMENT AND PERSONNEL

3.1 MANAGING THE ENVIRONMENT

Guideline 3

Laboratory management practices must aim to ensure the room and primary enclosures maintain the health and welfare of both the animals and personnel and provide consistency for research outcomes.

The most practical and effective way of providing suitable holding conditions for amphibians within an animal facility is to establish a set of general environmental conditions for the room(s) for such parameters as day length and temperature range. Each terrarium or aquarium should then be established as an individual environmental chamber in which temperature, light level, and humidity can be adjusted to suit the requirements of each species being held.

The micro-environment of each enclosure should meet the physiological needs of the species and life stage of the animals being held. Temperature and humidity requirements vary greatly; for example, some species from northern climates require cold temperatures for hibernation, while drying conditions are required for aestivation in other species (Browne et al., 2007). Additionally, for some species, adults may require very different conditions than younger animals (e.g., adults may require moderate humidity, while younger animals that have recently undergone metamorphosis may need very high humidity) (Browne et al., 2007).

Special equipment may be needed to maintain appropriate environments for amphibians. For example, a pharmaceutical fridge connected to an emergency power source may be necessary to over-winter animals, or humidifiers with filters changed regularly may be needed to maintain high humidity levels while preventing the growth of black algae.

Measures for a quick response to power outages must be in place to maintain appropriate environments for the amphibians being held.

3.1.1 Lighting

A variety of artificial lighting systems are available for amphibian housing; however, there is a lack of information on the effects of different spectral distributions on amphibians (Pough, 2007). Screening (aluminum or vinyl-coated fiberglass) in the enclosure lid allows direct transmission of light to the animals. Larger mesh size will enhance light transmission, but there must be assurance that the animals and any prey cannot escape (Browne et al., 2007).

The lighting spectrum and intensity must provide adequate support for normal health and behaviour. There is evidence that UV radiation or dietary vitamin D_3 contributes to health, reproduction, and immunity for some amphibians (Antwis and Browne, 2009; Ferrie et al., 2014; Verschooren et al., 2011). The benefits appear to depend on the species and natural habitat of the animals. Excessive UV radiation or the absence of a shelter has been reported to be detrimental to the skin and eyes of some species (Ferrie et al., 2014). Axolotls and other common laboratory amphibian species do not require UVB, and it may be detrimental for them. If UVB is used, it should be justified as being required by the model species (i.e., the default should be no UVB, as most amphibians do not require it). Where UVB is not used, and animals have health concerns related to skeletal development, UVB lighting or supplements should be considered.

Photoperiod influences the reproductive cycles of amphibians and the timing of metamorphosis (Pough, 2007). Therefore, the photoperiod for breeding programs should be similar to that found in the animal's natural habitat (Browne et al., 2007). Alteration of the photoperiod should be avoided when not related to breeding efforts or animal care committee-approved variables under study.

Xenopus should be housed with a 12/12 light/dark cycle (Reed, 2005; Schultz and Dawson, 2003; Green, 2010). Phasing the lights on and off is recommended to maximize reproductive success and fecundity (Reed, 2005; Schultz and Dawson, 2003; Green, 2010) and to avoid startling the frogs. This can be accomplished by having a lamp on a timer that turns the lamp on before and off after the main room lights. This is not necessary for axolotls as they do not appear to be startled by sudden changes in lighting.

For nocturnal species, the dark phase of the light cycle should provide low-intensity light to allow them to detect prey (Pough, 2007).

Rooms housing fossorial species should have the same lighting as rooms for other amphibians, but the animals must be provided with the opportunity to avoid the light through burrowing.

3.1.2 Temperature and Relative Humidity

3.1.2.1 Temperature

Temperatures should be determined by the needs of the species being held. Optimal temperatures vary markedly among and within species and with such divergent functional states as torpor, mating, feeding, and digestion.

In their natural habitat, amphibians control their body temperature by moving into a suitable thermal environment (Kreger, 2002). In the laboratory, it is often beneficial to provide temperature gradients within enclosures to allow this ability (Reed, 2005).

Temperature ranges in the species' natural habitat provide a good starting point for determining appropriate temperatures in the laboratory (Pough, 2007). Table 1 indicates temperature ranges suggested by Wright and Whitaker (2001). Pough (2007) suggests starting with lower temperatures (Table 2) and gradually increasing the temperature based on the animal's behaviour and appearance, as temperatures that are too high pose greater risk to the animals than temperatures that are too low. Behaviours that would indicate the need for an incremental temperature increase include inactivity, not feeding, and not producing feces (Pough, 2007). Dark-coloured appearance would also indicate the temperature is too cold (Pough, 2007).

Table 1 Temperature Ranges for Various Groups of Amphibians

AMPHIBIANS	CLIMATE	TEMPERATURE (°C)
Anurans	Temperate	16-24
	Tropical Lowland	22-28
	Tropical Highland	18-23
Salamanders		16-20
Caecilians		25-28

Table 2 Proposed Starting Points, With a Gradual Increase Based on the Animal's Response

NATURAL HABITAT	STARTING TEMPERATURE (°C)
Cold aquatic habitats	10
Cool temperate and montane tropical regions	15
Warm temperate and tropical environments	20-25

Seasonal changes in temperature and humidity, either rapid (occurring over a few days) or relatively slower, are essential for the maturation of ovaries and testes in some species. These seasonal changes are species specific, and captive breeding programs should provide seasonal cycles of the same magnitude and duration as those that occur in the animal's natural habitat (Browne and Zippel, 2007).

Some species undergo seasonal cycles that involve hibernation or aestivation. Frogs from northern areas have been maintained in the laboratory in a state of simulated winter torpor to eliminate their need to feed, as they will not usually feed on anything other than live, moving prey. However, this approach must be justified as it does not address the welfare of the animals and may result in poor performance of the animals in subsequent experimental procedures (Tinsley, 2010).

Frogs that are obtained from the wild in the spring and have recently emerged from torpor are beginning sexual maturity and have depleted body fat reserves. These animals will not usually survive further periods of cold and food deprivation.

When terrestrial amphibians are maintained under simulated winter torpor conditions, desiccation can be a serious problem, as air chilling units effectively condense most of the water vapor in the air. This can be compensated by ensuring the substrate in the enclosure is kept continually moist. A wide-mouth glass bottle with a few perforations in the lid to allow limited air circulation makes a suitable chamber for a terrestrial amphibian in simulated winter torpor. Moisture can be provided by placing pads of moist blotting paper on the bottom of the bottle and above the animal. Continuous darkness and a temperature of 1-4 °C should be maintained.

Leopard frogs that are collected in the fall can be maintained in a state of near torpor and held without feeding for four to five months. Maintaining this state requires a low light level, comparable to that at the bottom of a pond under snow and ice cover, with day length constant at 8-9 hours. Increasing day length and/or warming temperatures will induce the onset of sexual maturity, activate the frogs, and increase their food requirements.

3.1.2.1.1 Xenopus

The optimum temperature for *Xenopus laevis* is 18-22 °C (Reed, 2005; Tinsley, 2010); it should not fall below 16 °C or rise above 24 °C (Reed, 2005; Green, 2010). Stable temperatures within this range should be maintained. Abrupt changes in air or water temperature (i.e., within a period of hours) should be avoided (Reed, 2005). Temperature change greater than that experienced in their natural habitat (5 °C over 2-5 days) can result in thermal shock (Tinsley, 2010).

Xenopus tropicalis originate from warm areas, and adults should be housed at a water temperature of 24-25 °C; they do not do well at temperatures below 22 °C (Green, 2010). The development of tadpoles is temperature-dependent; a water temperature of 27 °C promotes growth (Kashiwagi et al., 2010).

Water temperatures below the optimal range for *Xenopus* may cause them to stop eating and negatively impact their metabolism and immune system (Reed, 2005). Temperatures above 30 °C are lethal to both species of *Xenopus* (Green, 2010).

Where chillers/heaters are not being used, it is important to monitor the room temperature; it should not fluctuate more than 6 °C over a 24-hour period (Green, 2010).

3.1.2.1.2 Axolotls

Axolotls should be maintained at a temperature of 16-21 °C. Below this range, the animals will stop feeding, and their metabolism and immune system will be depressed.

3.1.2.1.3 Larval Salamanders

Water temperatures of 10-12 °C are generally suitable for rearing larval salamander species found in Canada, and temperatures of 18-22 °C are suitable for most species of tadpoles. However, there are exceptions for some species or specific populations, and it is important to identify the animals properly and determine their requirements.

3.1.2.2 Humidity

Humidity requirements vary according to species. Some amphibians, particularly smaller species and those from humid environments (e.g., tropical rain forests), require nearly saturated humidity levels. For these animals, air circulation within enclosures should allow near-saturated humidity to develop, and the humidity should be monitored regularly. However, other species have lower rates of evaporation from the skin and can tolerate lower humidity levels (Withers et al., 1984).

Humidity requirements should be considered on a case-by-case basis. Where high humidity is required, the following methods may be useful:

- adding a porous paper cover over the ventilation ports to reduced air exchange and assist in humidity regulation;
- adding a container filled with damp peat moss to the enclosure;
- hanging an absorbent paper wick within the enclosure such that one end of the wick is in a dish of water; and
- using a sonicated fogger outside of the enclosure.

3.1.3 Air and Water Quality

3.1.3.1 Air Quality

Airflow in the room must be sufficient to allow surfaces to dry properly and to enable the exchange of oxygen and carbon dioxide through the animals' skin. The optimal turnover of air depends on the requirements of the species, the temperature and humidity of the source air, and the ability to create a microenvironment. A high turnover of dry air can lower humidity enough to dehydrate amphibians, while low turnover can result in increased humidity to a level that promotes condensation, microbial growth and contamination, and corrosion of metal.

For rooms housing aquatic species, a minimum of 12-15 air changes per hour, as recommended by aquatic equipment manufacturers, should be used as a starting point and monitored. Rooms housing terrestrial species may not require the air change rate to be as high as those housing aquatic species. Because pathogens can travel in aerosols over a distance of a few metres, any sick animals must be moved away from other animals beyond this distance.

3.1.3.2 Water Quality

Guideline 4

Water quality must be monitored regularly.

Water quality is very important to the health of aquatic animals and must be monitored in line with the capacity of the life support system. The frequency of monitoring should be based on the level of assurance that husbandry practices are adequate for maintaining good water quality for the particular animals present in the enclosure. The rate of water change in an enclosure should be based on maintaining water quality in relation to the animal's needs, which includes the development and maintenance of the skin microbiome.

It is important that the water in amphibian housing is free of chlorine and chloramine. Chlorine damages the protective mucous layer covering the skin of amphibians such as frogs and axolotls and can predispose these animals to infection (Reed, 2005). Chlorine and metals such as copper, which can leach from pipes, are toxic to some species and particular life stages.

The main water variables to be measured daily are temperature, pH, and conductivity/salinity. Measuring conductivity and/or salinity is essential for aquatic species; they will not survive in water treated with reverse osmosis if it has not been properly reconstituted.

When the system is being set up, frequent monitoring of other variables such as ammonia, nitrite, nitrate, and dissolved oxygen is necessary. Such monitoring should be in line with the capacity of the life support system and performed at least twice a week. Once the system is stable, monitoring frequency for these additional variables can be reduced to weekly, or possibly monthly, depending on the water change ratio, unless there is a change in the system, a large increase in amphibian density, or a problem is detected. When there is a health concern, an increase in density, or a problem with the equipment, there should be daily checks of water quality (Green, 2010), particularly nitrite, ammonia, nitrate, and alkalinity. If amphibians are housed in an environmental room with set temperature and humidity, the water temperature does not need to be monitored as regularly as in rooms without such environmental control.

General guidelines for water quality for amphibians provided by Wright and Whitaker (2001) are listed in Table 3. These are general ranges for amphibians, and requirements will vary based on the species, life stage, and location from which the animals originated. Departures from these general ranges should be justified with references.

Table 3 Ranges for Water Quality Parameters

PARAMETER	RANGE
pH	6.5-8.5
Salinity	0-5 ppt
Hardness	75-150 mg/L[5]
Alkalinity	15-50 mg/L[5]
Dissolved oxygen	>80% saturation
Carbon dioxide	<5 mg/L
Un-ionized ammonia	<0.02 mg/L
Nitrite	<1 mg/L
Nitrate	<50 mg/L
Chlorine	<0.01 mg/L[6]

3.1.3.2.1 Xenopus

Water quality parameters for *Xenopus laevis* are provided by Green (2010) and Sanders (2004, cited in Reed, 2005). While most parameters are similar to those listed for amphibians in general (see Table 3), recommendations for hardness differ, with Green recommending 175-300 mg/L and Sanders recommending 75-150 mg/L (as in Table 3). Both Green and Sanders recommend that alkalinity be 50-200 mg/L $CaCO_3$, which is higher than that noted in Table 3.

3.1.3.2.2 Axolotls

Municipal water must be treated to remove chlorine and chloramines, and it must be free of ammonia, chemicals, and heavy metals (Duhon, 2019). It is important that hard water is used, as the salts are necessary for the health of the axolotls' skin and the prevention of infections (Duhon, 2019). Alternatively, Holtfreter's or Steinberg's solutions can be used (Armstrong and Malacinski, 1989).

3.1.4 Sound and Vibration

As noted in Section 1, "Introduction", amphibians can hear in the range of 10 Hz-10 kHz (Heffner and Heffner, 2007; Capshaw and Soares, 2016), while humans are able to hear 20 Hz-20 kHz. In mammals, chronic levels of 'noise' at or above 85 dB have negative welfare impacts. In amphibians, a few studies have

5 Note that different ranges for hardness and alkalinity are given for *Xenopus* in the literature, and the information below should be consulted.
6 The method used must be capable of detecting chlorine at concentrations of less than 0.01 mg/L.

focused on anthropogenic noise, with noise levels at 70 or 87 dB; at these levels, there were negative impacts (e.g., high corticosterone levels) (Tennessen et al., 2014; Troïanowski et al., 2017). Generally, sound levels of ambient noise in nature (forested and open fields away from urbanized areas/roads) are lower (45-60 dB).

Research on the impact of sound and vibration on amphibians in both the field and in laboratories suggest that loud sounds result in breeding inhibition, increased vigilance, and increased levels of stress hormones (Morgan and Tromborg, 2007); however, research in this area is limited and the effects may be species specific (Simmons and Narins, 2018). It should also be noted that sound travels faster in water than air, which could have further implications for aquatic amphibians.

The level of vibration in the facility should be assessed (particularly when renovations are taking place) and considered a potential negative welfare concern. Within enclosures, measures such as insulating vibrating filters from the holding tank should be considered. Where the source of vibration is outside of the enclosure, potential solutions include placing rubber under tanks or standing racks in buckets of sand.

3.2 PEST AND VERMIN CONTROL

General information on pest and vermin control is provided in the *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017); also see Section 6.7, "Cleaning and Sanitation", for more specific information on enclosure disinfection for amphibians. Proper storage of amphibian food is important to prevent the presence of pests and vermin.

Control of insect pests in facilities housing amphibians should be done through physical methods and should not involve the use of chemicals. Amphibians are very sensitive to insecticides, and insect infestations within enclosures should be managed with physical methods, such as sticky paper that is placed out of the reach of the animals.

If an insecticide is used, extreme care must be taken to prevent exposure to any insect that may be consumed by an amphibian.

3.3 PERSONNEL

Guideline 5

Amphibians must be observed regularly by trained personnel, with minimal disruption to the animals.

Sufficient animal care personnel are needed to ensure: 1) enclosures are cleaned, food and water are provided, and other husbandry requirements are addressed, as appropriate; and 2) animals are observed regularly. Amphibians must be observed by trained personnel who can recognize welfare concerns and health problems in that species and resolve them through institutional standard operating procedures (SOPs). Proper record-keeping and reporting procedures must be followed to ensure the facility manager and veterinarian remain informed, and investigators are alerted to any changes (see the *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017)). The frequency of observation should be described in an SOP for each species, taking into account seasonal changes such as hibernation.

For species that hide, disturbance to the animals caused by digging them up or removing their cover to facilitate observation must be balanced with the need to confirm their health. However, all systems supporting the animals (containment and environmental) and the conditions in the macro-environment must be checked daily, and ideally alarmed, so that systems failures are notified to animal care personnel immediately to avoid stress or death to the animals.

All personnel should use appropriate practices that respect the welfare of the animals (e.g., not tapping on the tank and moving tanks in a way that minimizes disturbance). Checklists are useful to facilitate consistent and objective observation of the animals over time by multiple personnel. These lists should be kept in the room with the animals.

Where welfare concerns are identified, any additional demands on personnel time to implement appropriate mitigation strategies need to be considered and accommodated.

4 PROCUREMENT

4.1 SOURCE

For reasons of animal health and welfare, and the quality of science, amphibians should be obtained from captive colonies and bred specifically for research purposes. These animals have a known life history, age, and diet, and the potential for introducing unwanted disease or parasites to an existing colony can be reduced. They have also been reared in artificial housing and husbandry conditions, and therefore, less acclimation to the laboratory environment is required compared to wild-caught animals. For example, laboratory-bred *Xenopus* have been noted to habituate to people moving in the room and not react to sudden loud noise or husbandry practices (Council of Europe, 2003). In addition, taking animals from the wild can have an impact on the local ecosystem.

There are some studies that may require wild-caught amphibians. For example, wild-caught *Xenopus* may be needed because of their higher fecundity than laboratory-bred counterparts, which results in fewer animals to obtain a required amount of biological material (Council of Europe, 2003), or they may be needed to maintain genetic diversity in a colony (Reed, 2005). See Section 4.2, "Regulations", for regulations concerning wild-caught amphibians.

4.2 REGULATIONS

Guideline 6

Facilities and investigators acquiring, transporting, or conducting research on amphibians must be familiar with, and comply with relevant international, federal, and provincial/territorial legislation and policies.

In addition to provincial/territorial regulations, investigators need to determine other relevant regulations and permits. For example, where population status is a concern, a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit may be required, and for requirements regarding pathogen control, the Canadian Food Inspection Agency (CFIA) is the point of contact.

Requirements for CITES-listed species are available through Environment and Climate Change Canada. The CFIA's Automated Import Reference System (AIRS) is a useful resource for determining regulations for imported animals.

Importation of any specimen of the order Caudata (i.e., salamanders, newts, and mudpuppies) requires a permit issued by the Minister of the Environment (Government of Canada: Salamander permitting policy).

Where wild capture of amphibians is not covered by provincial/territorial regulations, animal care committees should evaluate the impact on the ecosystem and ensure protocols describe measures to reduce the impact, such as cleaning equipment between sites and only taking a portion of each egg mass (see Section 5.4, "Eggs").

4.3 PRE-SHIPMENT PROCEDURES

Guideline 7

Information relating to the transport, welfare, and care of the amphibians must be communicated between the supplier and receiver before shipment of the amphibians takes place.

Health documentation must be provided in accordance with regulations governing the movement of animals. See the Canadian Food Inspection Agency (CFIA) for documentation requirements. For international air transportation, International Air Transport Association (IATA) requires shippers to confirm in writing that the animals are healthy. Proper documentation is important to avoid delays in transporting animals.

All available documentation on the health of the animals, the conditions under which they have previously been housed (i.e., temperature, humidity, food, husbandry practices, enrichment, etc.), any special needs, and the transport conditions will assist in addressing the needs of these animals during transport, should there be an interruption in transport, and upon arrival.

4.4 TRANSPORTATION

Amphibians should be transported during seasons with moderate temperatures relative to their natural habitat. If it is necessary to transport them during winter or summer months, precautions must be taken to maintain the temperature in the container within an acceptable range for that species (e.g., by adding ice packets or chemical heat packs to the secondary container) (Reed, 2005). Transportation should be postponed if extreme weather conditions could threaten the welfare of the animals (Reed, 2005).

Transport routes should be of the shortest duration practical. Potential sources of delays in transit should be anticipated, with plans made to protect the welfare of the animals delays they occur (Reed, 2005).

The IATA recommendations for animal transportation must be followed for air transportation. They also provide useful information for land transportation.

Amphibians should be transported within two distinct types of containment: a primary container to house and contain the animal, which is placed within a rigid outer secondary container for protection. Lids or openings of primary containers must be closed tightly and securely sealed. Crushed newspaper or foam packing chips can be used to support the primary container within the secondary container and prevent jarring during transport. Primary containers must be kept within the acceptable temperature range for that species and out of direct sunlight.

The primary container must be made of water-resistant material that will not degrade when wet, as amphibians need to be kept moist. When cloth bags are used as the primary container, the bags must be kept wet; when plastic containers are used, a damp substrate must be provided. The primary container must have an adequate air supply, and caution must be taken to ensure the weight of the wet bag or substrate does not smother the animal and there are no loose objects within the container that could harm the animal. Some amphibians may also be transported in a sealed plastic bag containing water and blown full of air. The bag must be sufficiently strong and placed in a secondary container that does not have any surfaces that could puncture the bag.

Damp cloth bags that are used as primary containers must not be made of abrasive material such as burlap. If plastic or Styrofoam containers are used, they must be new or thoroughly washed, rinsed, and allowed to air dry before reuse. Small holes (3-6 mm) need to be made through the sides and tops of the containers for ventilation. The holes should be made from the inside out to avoid creating sharp edges on the inside that could cause abrasions on the animals. A cushioning substrate should be placed in these containers to reduce traumatic injuries and to provide a water source for the animal in transit. However, care must be taken to ensure the substrate is not abrasive, as this would damage the sensitive skin of amphibians. Slightly dampened inert material that is pulled apart to create air spaces and refuges for the animals provides a good packing medium. The material must not be saturated with water to avoid crushing, trapping, or drowning the animals. Pieces of moistened sponge can provide a suitable substrate. Dampened paper towel should not be used since it does not provide protective cushioning.

The size of the packing container depends on the size and activity level of the amphibian being shipped. Anurans tend to jump and collide with the container during shipping, causing injury to themselves. To prevent injury, their movement should be restricted by limiting the height clearance in the primary container.

To prevent cannibalism, inter-species aggression, and toxic effects between certain species, different size classes (i.e., small and medium) and different species should not be mixed in the same packing container.

For all amphibians, regardless of their size, natural habitat, or the number of amphibians transported together, the primary container must be of sufficient size to allow the entire ventral surface of each animal to make contact with the bottom of the container. The following additional recommendations should be used to determine container sizes:

- Large amphibians (anurans with a snout-vent length over 15 cm and other amphibians with a total length over 30 cm) should be transported individually. The primary container should be a minimum of 5 L in size. Cloth bags should be a minimum of 30 x 45 cm;
- Medium amphibians (anurans with a snout-vent length of 6-15 cm and other amphibians with a total length of 15-30 cm) can be transported in the same container, up to a maximum of 20 animals per container. Each animal should have a minimum of 250 mL of space. Cloth bags should be a minimum of 30 x 45 cm;
- Small amphibians (anurans with a snout-vent length of 3-6 cm and other amphibians with a total length of 6-15 cm) can be transported together to a maximum of 40 animals per container. Each animal should have a minimum of 100 mL of space. Cloth bags should be a minimum of 30 x 45 cm; and
- Very small amphibians (anurans with a snout-vent length less than 3 cm and other amphibians with a total length less than 6 cm) can be transported together to a maximum of 50 per container. Each animal should have a minimum of 50 mL of space. Cloth bags are not recommended for very small amphibians.

Amphibian larvae must be transported in water that is sufficiently aerated. This could be accomplished using a blown-up bag that contains water and air in proportions determined by the needs of the animals and the risk of splashing, which can harm the animals.

Insulated foam secondary containers (a Styrofoam inner box placed in an outer cardboard box) are recommended to prevent sudden changes in temperature and to provide a buffer against temperature extremes. However, Styrofoam is porous and will not hold water. It is imperative to ensure internal plastic bags and containers do not leak, as any leaked water will escape the Styrofoam box and soak the external cardboard, which could cause transport delays. Boxes constructed from water-resistant fibreboard or plywood and lined with insulating foam or polystyrene can also be used. Heat packs and cold packs may be placed inside the insulated shipping box to compensate for the external environment; however, these are of limited value and cannot be relied upon if the container and animals are to be exposed to extreme temperatures for an extended period. If the temperature is a concern, temperature loggers can be placed within the shipping container to indicate if there has been any temperature stress for the animals.

If notably toxic species (e.g., *Rana palustris* (pickerel frog) and *Taricha spp* (western newts)) are transported, measures must be taken to ensure good water quality is maintained, for example, by changing the water if there is a long holding period.

4.4.1 *Xenopus*

Transportation and re-housing are stressful for *Xenopus* and may result in increased corticosterone and decreased body mass for up to 35 days (Holmes et al., 2018). Excessive heat is a significant concern during transport; temperatures above 25 °C may lead to skin cell disintegration and can damage eggs within females (Reed, 2005).

Sphagnum moss and foam cubes have been used as a substrate for transport. In general, sphagnum moss works well for this purpose, but there have been indications that it can be abrasive (Holmes et al., 2018) or susceptible to mite infestation (Ford et al., 2004).

4.4.2 Larvae

For *Xenopus*, Green (2010) suggests transporting larvae in bags filled with 2/3 air and 1/3 water.

Small axolotls (2-3 cm) can be maintained in a healthy condition with low risk of harm from splashing when transported in bags with 90% water and 10% air that are kept at 15-16 °C.

4.5 RECEIVING ANIMALS

Prior to receiving amphibians, the enclosures to which they will be transferred should be checked to ensure the water temperature and water quality variables (pH, conductivity, nitrate, nitrites, etc.) are within acceptable ranges (see Section 3.1.3.2, “Water Quality”).

Facilities should have procedures in place for the transfer of amphibians to enclosures specific to the species and life stages that will be received. These procedures should include the number of animals to be transferred into each enclosure.

To reduce the risk of spreading disease or bacteria, the water and any substrate in the transport containers should not be transferred to the new holding tanks (Reed, 2005). In addition, the animals should be dipped in a separate static tank of water to rinse off any packing material or residue and then transferred into the designated enclosure. The veterinarian must be consulted with regard to any proposed use of antibiotics or antifungal agents.

Guideline 8

The health and welfare of amphibians must be checked upon arrival by competent animal care personnel.

The health and welfare assessment of the animals upon arrival should be described in a SOP and include verifying that the animals received correspond to the order, ensuring all animals have been removed from the transport container, and visually inspecting the animals' condition. Where practical, the animals should be weighed and measured, and fecal samples should be tested for parasites. Information received from the supplier (see Section 4.3, "Pre-Shipment Procedures") should be used to determine the quarantine procedures.

Animals showing signs of illness or injury should be separated from others in the shipment and assessed by a veterinarian to determine the appropriate course of action (e.g., monitoring, treatment, or euthanasia). Any animals that are dead upon arrival should be removed from the container, and a post mortem should be conducted as soon as practical.

It is important to provide newly arrived amphibians with substrates similar to those they have previously been exposed to. For wild-caught amphibians, substrates from their natural environment can be sanitized by heating to >70 °C for 20 minutes (Browne et al., 2007).

4.6 QUARANTINE AND ACCLIMATION AFTER RECEIPT OF ANIMALS

Guideline 9

Amphibians should undergo a period of quarantine and acclimation after transport and before use in studies.

Quarantine is important to prevent the spread of pathogens (such as chytridiomycosis) from surrounding environments into the facility, within the facility, and from the facility to surrounding environments (Browne et al., 2007). Quarantine should ideally occur in a separate room; where this is not possible, measures that prevent splashing and aerosol transfer between enclosures (e.g., on separate shelves) must be taken. To protect the resident animals, husbandry procedures for amphibians in quarantine should be undertaken after the other animals in the facility, and equipment (including personal protective equipment) must be dedicated to the quarantine area (Reed, 2005). Where different groups of animals are in quarantine, precautions should be taken to prevent the transmission of pathogens between groups.

Facilities should have the capacity for full quarantine, including the water supply and the movement of personnel and materials (e.g., substrates for enclosures). Quarantine areas should be cleaned frequently, with floors, surfaces, and drains sanitized.

All quarantine enclosures must be disinfected after use, and the water must be treated. Bleach is effective for disinfecting surfaces, with the bleach to water ratio and required contact time based on the expected presence of pathogens; for examples, see Poole and Grow (2012), CDC (2017), and CFIA (2018). It is important that residues are removed through rinsing and that surfaces are dry before use. When possible, enclosures and equipment should be autoclaved.

During quarantine, amphibians should be observed daily for signs of ill health (Reed, 2005), such as skin lesions, pallor, cachexia, and abnormal eating habits (see Section 9, "Welfare Assessment"). Quarantine procedures will depend on the source of the amphibians, but all suspect amphibians should be tested for chytridiomycosis (Boyle et al., 2004). Any treatments should be provided in consultation with the veterinarian.

The quarantine period should be at least as long as prescribed by the veterinarian, based on the species and location. Depending on the type of study and health surveillance practices, wild-caught amphibians should generally be held for a quarantine period of at least 90 days (Wright and Whitaker, 2001). Captive-bred amphibians with known health status generally require a shorter quarantine period (O'Rourke, 2002, cited in Reed, 2005), with the length depending on fecal analyses and physical evaluations.

A period of acclimatization, which can run concurrently with the quarantine period, is important to ensure any stress associated with transportation has been alleviated and the physiology of the animal has returned to a normal state. The length of time required will depend on the conditions of transport, age of the animals, the particular animals, and the studies they will be involved in. During this period, the animals should be habituated to the environmental conditions and husbandry procedures, such as the provision of food and water. Animals should be acclimated to the relevant study conditions and procedures to reduce stress when the studies begin.

4.6.1 *Xenopus*

The recommended minimum period of quarantine for captive-bred *Xenopus* ranges from 7-10 days to 30 days; however, the major parasites of *Xenopus laevis* have lifecycles that last 4-6 weeks, and some advise that quarantine should cover this period (Sanders, 2004, cited in Reed, 2005). A period of 90 days is recommended for *Xenopus* taken from the wild (Reed, 2005).

During quarantine, daily observation of *Xenopus* includes looking for signs of ill health, such as changes in activity level, skin discoloration, ulceration and hemorrhages on the legs, or coelomic swelling (Reed, 2005).

4.6.2 Axolotls

Captive-bred axolotls should be quarantined for a minimum of 7-10 days. A longer period may be necessary at the discretion of the veterinarian.

5 BREEDING

There is considerable variation in reproduction among amphibians, and the lack of laboratory-bred strains is partly due to the difficulty of establishing suitable environmental conditions to elicit sexual maturity and mating behaviour. Even when reproduction can be achieved, it is difficult to successfully rear many amphibians through larval and juvenile stages (Browne and Zippel, 2007).

For some species, the simulation of natural reproductive cues promotes breeding in captivity. For example, newts will breed successfully in captivity without hormone treatments when the temperature is set below that at which they are normally housed for 2-3 months. However, for other species. hormonal treatments are required to induce breeding (Trudeau et al., 2010). Both natural and artificial reproduction are therefore important to the long-term management of captive amphibians (Browne and Zippel, 2007).

Browne and Zippel (2007) discuss hormonal triggers and changes necessary for natural breeding. For successful breeding, environmental conditions should replicate those found in the animal's natural environment, for example, temperature (including temperature changes and temperature ranges that are conducive to hibernation and aestivation), food, and photoperiod (Browne and Zippel, 2007). For some species, vocalizations are also important to initiate breeding.

Brown and Zippel (2007) provide a review of the use of hormone injections for the induction of oocytes or sperm and techniques for the collection and preservation of viable eggs and sperm.

5.1 BREEDING AGE AND CONDITION

Guideline 10

Species-specific health assessment benchmarks should be established for breeding animals, and these should be met prior to initiating breeding.

Many amphibian species display sexual dimorphism. For example, male and female anurans can be identified by nuptial pads, tympanum size and colour, throat and body colour, and vocalizations (Browne and Zippel, 2007). Breeding ages and frequencies vary among amphibians, but in general, males reach sexual maturity at a younger age than females (Browne and Zippel, 2007), and the number of eggs produced increases with the weight of the female (Reed, 2005).

5.1.1 *Xenopus*

Xenopus laevis females can be bred between the ages of one and five years, with peak production occurring at 2-3 years of age (Green, 2010). They should not be bred more than once every 1-3 months, and ideally, the rest period between breeding should be 6 months (Green, 2010). Males can be bred up to 3 years of age at a frequency of 2-3 times per month (Green, 2010).

For *Xenopus tropicalis*, females can be bred as of 6-9 months of age, with breeding occurring every 4-6 months and a maximum breeding age of 5-8 years (Green, 2010). The age requirements for breeding males are similar.

Animals can continue to be used for breeding past these times if they are in good health and good quality eggs and embryos continue to be produced.

5.1.2 Axolotls

The minimum breeding age for females is 12 months, while males can be bred at 9-12 months (Khattak et al., 2014; Duhon, 2019). Both females and males can be bred up until 7-8 years of age; beyond this age, reproduction declines. Females can be bred every 2 months (Khattak et al., 2014; Duhon, 2019), but preferably every 3 months. Males should be bred once every 4 weeks (Khattak et al., 2014); however, more frequent breeding (up to every 1-2 weeks) may occur if the animals remain healthy and productive (Duhon, 2019).

5.2 CONSIDERATIONS FOR BREEDING MANAGEMENT

Breeding strategies among amphibians include external and internal fertilization: anurans are nearly all external fertilizers, while caecilians and many salamanders and newts are internal fertilizers (Kupfer et al., 2004; Kikuyama et al., 2009). Amphibians also range from oviparous to viviparous species and include some species where females have specialized pouches in which eggs and larvae are carried. The number of eggs varies considerably among oviparous species; for example, within anurans, *Lithobates pipiens* lay approximately 2500 eggs, while *L. catesbeiana* (bullfrog) can lay 20,000 eggs (Gilbert, 2000). Larvae of most species are aquatic; however, salamanders of the genera *Plethodon*, *Ensatina* and *Aneide*, deposit eggs in protected, moist microenvironments on land, and terrestrial juveniles emerge from the eggs.

In the laboratory, females and males should be housed separately prior to breeding. Enclosures for breeding must be of a size appropriate for the species and have a suitable depth of water and enrichment. For natural breeding to occur, the environment must promote natural behaviours.

When using air conditioners or refrigeration units to reduce the temperature for a specific period to prime amphibians, there must be assurance that adequate moisture levels are maintained. The technology used to cool the air often removes moisture.

5.2.1 *Xenopus*

As with other amphibians, male and female *Xenopus* should be separated prior to mating to induce breeding. For *Xenopus laevis*, appropriate temperature, good water quality, and enrichment (in the form of an artificial lily pad) have been found to positively affect oocyte and embryo production (Heyworth and Owens, 2019). For *Xenopus tropicalis*, adult frogs should be maintained at 22–24 °C for the production of embryos for scientific studies; higher temperatures negatively impact both amplexus and spawning rate (Kashiwagi et al., 2010).

5.2.2 Axolotls

Adult axolotls are often housed singly prior to mating; group housing is possible but may result in decreased mating efficiency (Khattak et al., 2014). Females in good breeding condition have a round belly, which indicates the presence of eggs, and males are muscular and healthy, with a well-shaped cloaca

(Khattak et al., 2014). Mating tanks are kept at a lower temperature (15-16 °C) than the regular group holding tanks (18-20 °C) (Khattak et al., 2014).

For mating, the male and female are put into the mating tank along with plastic leaves or rocks, and the water flow is shut off (Khattak et al., 2014). Ice cubes made with dechlorinated water can be added to the water to reduce the temperature to 11-12 °C over a 24-hour period, which stimulates the production of spermatophores. The success of a particular pairing is unpredictable, but most pairings should produce spermatophores (Duhon, 2019).

5.3 ARTIFICIAL INDUCTION OF BREEDING

Inducing sexual maturation and ovulation or sperm emission in anurans (chiefly *Xenopus laevis* and *Lithobates (Rana)* spp.) by injection with frog pituitary gonadotropin or mammalian chorionic gonadotropin has been in use for some time. More recent advancements in hormonal therapy are discussed by Trudeau et al. (2010). Amphibians brought to maturity in this way usually need to be artificially spawned, as they do not necessarily show mating behaviour. Browne et al. (2001) describe methods to extend the period over which oocytes remain viable using low temperatures or saline solutions to increase osmolarity and then storing them in simplified amphibian's Ringer (SAR) or DeBoer's solution.

Species-specific breeding protocols should be consulted to determine appropriate hormone doses, water levels, and pH. When amphibians are injected with hormones to induce breeding, each needle should only be used once as they dull very quickly and could harm the animals.

For surgical removal of oocytes, see Section 10.9, "Collection of Oocytes".

5.3.1 *Xenopus*

Xenopus are normally primed (injected with gonadotropin) to trigger the maturation of oocytes and induce ovulation (Green, 2010; Wlizla et al., 2017). When collecting eggs from a primed female by stroking the lower coelomic cavity, care must be taken to ensure the pressure does not cause trauma to any muscles or internal organs (Green, 2010). Oocytes can also be harvested surgically under general anesthesia (Green, 2010).

For breeding *Xenopus tropicalis*, the water should be at pH 5.8-6.0. Females are injected with gonadotropin and kept separate from the males for the first day. On the second day, mating pairs are housed together, and clasping should take place within 2-6 hours. Egg collection with a very fine mesh net should begin after 3-4 hours of amplexus to prevent eggs from being eaten. Eggs should be transferred to Petri dishes at a density of no more than 100 eggs per dish. After breeding, the male and female should be moved to separate holding tanks and isolated until the next day to monitor their health and ensure the female has completed egg-laying (Showell and Conlon, 2009).

5.3.2 Axolotls

Healthy axolotls are not difficult to get to spawn, and hormone injections are generally not required (Duhon, 2019). Animals should be given a rest period of two months after spawning. There may be instances where animals do not spawn following the rest period, and hormones are needed. In such cases, gonadotropin is used, as in frogs.

5.4 EGGS

If eggs are sourced from the wild, the number of eggs collected should be based on the egg mass size, the abundance of egg masses at the site, and the ecological threats. Only a portion of each egg mass should be taken to minimize potential impacts on wild populations.

If adults in amplexus are captured in the wild with the intent of having them lay eggs in containers and subsequently be released, those animals must be released at their original capture site. Animals brought into the laboratory should not be returned to the natural environment to avoid potential disease transmission.

Egg jelly provides defense against water mold, and the decision to remove the egg jelly should be based on the species and environment (Gomez-Mestre et al., 2006). The procedures for removing egg jelly are also species specific; for example, L-cysteine wash is effective for some species but does not work on axolotl eggs. In some cases, removing the jelly and separating eggs may reduce the spread of mold between infected and healthy eggs. Once the jelly has been removed, the eggs should be kept in solution (e.g., Ringer's solution for eggs of Canadian species or Frog Embryo Teratogenesis Assay-Xenopus (FETAX) solution[7] for *Xenopus* eggs). Additional care following the removal of the jelly will depend on the species (e.g., for *Xenopus*, see Wlizla et al., 2018). For axolotls, there are no chemicals used in the removal of jelly, and since the eggs are not taken from the wild, there are no disease concerns.

Eggs should be kept in conditions that mimic their natural environment in terms of temperature, shade or no shade, water flow, etc. The density should be based on the stage of development (e.g., <100 *Xenopus* embryos before stage 35/36 for a 10 cm petri dish and <50 embryos at stage 35/36 for the same size dish (Wlizla et al., 2018)). Eggs should be checked daily, and any that are found to be dead should be removed.

5.5 CARE OF OFFSPRING

Mortality rates for young amphibians in captivity should be lower than in the wild; however, two stages have a particularly high risk of mortality, even in captivity: hatching and the transition from the tadpole/larval form to the young adult form. Daily monitoring and removing dead embryos is important to maintain healthy offspring. Animals should also be carefully monitored to anticipate their changing husbandry needs, such as the type and amount of food and the type of enclosure required to meet their needs and prevent escape. For species that transition from an aquatic to terrestrial form, measures must be taken to prevent drowning. For example, prior to metamorphosis, floating pads could be added, or the tank could be tilted to produce a dry area.

If newly hatched larvae are to be moved, plastic pipettes should be used. Soft, fine mesh nets can also be successfully used, depending on the size of the larvae. Latex gloves should not be used.

Procedures for the care of offspring depend on the species. For some species of anurans, high-density rearing systems, described by Browne et al. (2003), can be appropriate for raising larvae to metamorphosis.

Salamander larvae are cannibalistic and should be singly housed. If housed together, they become aggressive if there is competition with other larvae for food. Securing a regular source of food items small enough

7 ASTM E1439-12(2019), *Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)*, ASTM International, West Conshohocken, PA.

for the terrestrial form as it grows to a mature size is important (e.g., pinhead crickets, wax worms, and drosophila (flightless if accessible)). Gut loading or dusting of food items with supplementary calcium, vitamins, etc., is recommended. If gut loading, insects need to be fed immediately, or the supplements will be metabolized.

Neonatal caecilians, except *Typhlonectes*, which is fully aquatic, have similar environmental and nutritional requirements to those of adults (Browne and Zippel, 2007). They undergo metamorphosis and lose their gills close to the time of birth, and therefore gravid females should be kept in shallow water (3 cm) (Browne and Zippel, 2007).

5.5.1 *Xenopus*

Tadpoles should be fed a suitable food source (e.g., powdered tadpole food or spirulina) when they reach stage 37/38[8] (5-10 days post-fertilization) (Green, 2010). The frequency of feeding should be a minimum of 2 times per week, with monitoring to avoid overfeeding, which degrades the water quality. Once limb buds form (stage 65/66), the animals should gradually be introduced to adult pellets (Green, 2010). The rate of development of tadpoles is influenced by both the temperature and the amount of food provided.

Tadpoles should be housed with others of similar size (Green, 2010). An initial stocking density of 50 tadpoles per litre should decrease as the animals increase in size and maturity. The density should be five animals per litre at the onset of metamorphosis and approximately one animal per 4 L once limb buds form (Green, 2010).

Froglets should be provided with the opportunity to hide and to rest on surface objects, as they are not strong swimmers and can drown from exhaustion (Chum et al., 2013).

Daily water changes of 25-50% of the water volume should be performed once the tadpoles are at stage 55 (Green, 2010). As they approach metamorphosis, measures must be taken to prevent escape.

Tadpoles should be handled by scooping them with containers rather than nets to avoid injury (Green, 2010).

5.5.2 Axolotls

Axolotl larvae need to be size-sorted as soon as they begin to feed to prevent them from eating each other.

Larvae (from hatching until 5 cm from snout to tail tip) are fed a daily diet of live *Artemia* (brine shrimp) that have been separated from their shells (Khattak et al., 2014; Duhon, 2019). Care should be taken to avoid feeding unhatched eggs or the shells of hatched eggs, as they could cause intestinal blockage if consumed or foul the water if left uneaten. Juveniles (6-20 cm) are fed *Artemia*, frozen brine shrimps, bloodworms, and/or small fish pellets (up to 3 mm) every day. As juveniles grow, the amount of *Artemia* should be reduced until they adapt to fish pellets only (Khattak et al., 2014). If a problem arises, it may be due to feeding pellets too early.

8 Based on Nieuwkoop and Faber (1967).

Larvae are susceptible to disease and should be fed a sufficient amount while maintaining good water quality, and they should not be held in overcrowded conditions (Duhon, 2019). The amount of food provided should fill the animals to satiation, without leaving excess food in the water (Duhon, 2019). Water should be changed 2-4 hours after feeding, as uneaten shrimp cause rapid deterioration of water quality. Upon hatching, young larvae can be kept in shallow glass bowls at a density of 50 or more animals per 20 cm diameter bowl (Duhon, 2019). As the larvae grow, the density should be reduced and the animals sorted by size (Duhon, 2019). The rate of growth depends on the temperature, frequency and amount of food, and the number of animals per bowl; larvae should be approximately 2.5 cm long when 1.5-2 months old (Duhon, 2019).

6 HUSBANDRY

The *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017) should be consulted for general guidelines that apply to all species. This section provides additional considerations that are important for amphibians.

Housing and husbandry should simulate the natural conditions of the species, taking into account the welfare of the animals and the need for personnel to observe them (Tinsley, 2010).

6.1 IDENTIFICATION

All animal enclosures should be clearly marked, as described in the *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017), Section 2, "Identification of Animals". The need for individual identification of animals should be justified, and the least invasive method suited to the study goals should be used.

The preferred methods of identification for amphibians are natural markings and visible implant elastomer (VIE). Other methods (e.g., microchip transponders; attachment of plastic beads, studs, or tags; injected decimal coded wire tags; and radioactive markers) may be suitable in some circumstances. See Appendix 2 for information on the advantages and disadvantages of various methods of identification.

6.2 ANIMAL OBSERVATION

As mentioned in Section 3.3, "Personnel", amphibians must be observed regularly by trained personnel, and the frequency of observation should be described in an SOP for each species. For species that hide, disturbance to the animals caused by digging them up or removing their cover to facilitate observation must be balanced with the need to confirm their health. However, all systems supporting the animals (containment and environmental) must be checked daily.

6.3 HOUSING MANAGEMENT

Many amphibians can be group-housed by species; however, they should be sorted by size, as larger animals may try to eat smaller ones. In the case of larvae, care should be taken to avoid overcrowding, which can stunt their growth.

6.3.1 *Xenopus*

Frogs should be housed in stable groups with other frogs of similar size (Reed, 2005). *Xenopus laevis* naturally form hierarchies, and group stability is important (Reed, 2005).

While meeting the density requirements for frogs described in Section 2.2.1, "Spatial Requirements", there should be a minimum of 5-6 animals per tank to promote feeding (Reed, 2005; Green, 2010). When desirable, efficient feeding can involve feeding frenzies, which are less prevalent when fewer frogs are housed together

(Reed, 2005). However, overcrowding can result in traumatic injuries during feeding frenzies and should be avoided (Reed, 2005).

There is little evidence for ideal group size, but once the number of animals necessary to stimulate feeding behaviours is reached, density becomes important (Reed, 2005). Hilken et al. (1995, cited in Reed, 2005) found that frogs provided with more area per animal grew significantly faster than animals kept in a tank with a higher population density. Overcrowding and insufficient water levels have also been found to affect oocyte quality in females by causing ovary regression (Alexander and Bellerby, 1938, cited in Reed, 2005).

The density is appropriate when there is no injury, cannibalism, or build-up of waste materials, and the frogs maintain a good body condition score. The holding system will influence the optimal density; a higher density of frogs can be maintained in a flow-through system than a static system. However, animals of some species do not like water movement, and flow rate should be set accordingly.

6.3.2 Axolotls

Axolotls are commonly housed individually when not being mated, as this results in better breeding success than when group-housed (Duhon, 2019). In addition, axolotls feed by reflex and routinely bite each other when group-housed. This behaviour starts at an early age when axolotls begin to feed.

Adult axolotls can be housed individually in 4 L bowls or in groups of 3-4 in plastic tubs (25 cm x 46 cm x 15 cm) (Duhon, 2019). They have also been housed successfully in filtered aquaria (two adults in a ten-gallon aquarium) (Duhon, 2019), but there should be assurance that the water flow is not excessive. A water volume of 2 L per axolotl is adequate, providing the animals can completely submerge and the water is changed frequently (Duhon, 2019). Axolotls should be provided with the option to hide unless it interferes with the study.

Larvae require much smaller volumes; for example, a 3 cm axolotl can be kept in 50 ml of water, and a 10 cm juvenile can be kept in 200-300 ml of water, providing the dimensions at least meet the minimum performance standards indicated in Section 2.2.1, "Spatial Requirements".

6.4 NUTRITION, FEEDING, AND WATER

Amphibians generally exhibit narrow feeding adaptations, and most feed in response to the movement of prey (Pough, 2007). It is important to search the relevant literature on the nutritional requirements of each species to be maintained in captivity. However, for many species, there is limited information available and there is a risk of nutritional deficiencies in individuals maintained on diets that differ from their natural foods. In general, captive amphibians should either be maintained on their natural foods or foodstuffs closely approximating their natural diets. However, the food supply should come from a reliable and regular source to prevent the introduction of pathogens and other variables.

Insects are a common food for amphibians in captivity. To provide a nutritionally complete diet, a variety of types of insects should be fed, along with vitamin and mineral supplements (Pough, 2007). Vitamins and minerals can be dusted onto an insect just before it is fed, or they can be included in the food of the insects (Pough, 2007).

When institutions obtain frozen food items, care must be taken to avoid contamination during thawing and refreezing. Only high-quality frozen food should be purchased, and it should be repackaged in small quantities, with the time before refreezing minimized. Frozen food should not be left out in the room to thaw.

When introducing amphibians to non-living food items, it is important to be aware that some species require extensive conditioning to elicit a feeding response, and their nutritional reserves may become dangerously depleted during the process.

Daily feeding for adult amphibians is usually not necessary; in most cases, food should be provided 1-3 times per week. Amphibians should be fed to satiation at each feeding.

Adult anurans are predators, and in their natural habitat, they consume foods ranging from earthworms, slugs, crickets, and insect larvae to various flying insects (preferred by frogs) and other vertebrates (for large leopard frogs and bullfrogs). In captivity, toads are among the easiest amphibians to feed, as they will eat a wide variety of worms, slugs, crickets, and insect larvae, which are readily obtainable year-round. Flightless fruit flies are suitable food for metamorphs of some species, such as the American toad.

Most adult *Lithobates (Rana)* spp. will only feed on flying insects or other active prey. Multiple food types (e.g., crickets, worms, and flies) should be offered to stimulate feeding. For some individuals, feeding behaviour may be stimulated by dangling a non-living food item in front of them; however, many frogs are disturbed by human presence and will not feed. Amphibians should only be force-fed in emergency situations.

Most larval anurans are herbivores or detritivores, feeding on aquatic algae, higher plants, and organic debris in the substrate. In captivity, they will feed on pieces of leafy green vegetables that are softened by boiling. However, these vegetables vary in nutritional content and should be selected based on their nutritional value for the animals (Kohman, 1939). For some species, spinach can cause lethal deposits of calcium oxalate crystals in the kidneys (Briggs, 1941; Forzán et al., 2015). Larval anurans can also be fed dried commercial algal pellets and most dry, commercially prepared aquarium fish foods. There are a few species in which tadpoles are predatory (e.g., *Scaphiopus bombifrons* and *S. intermontanus*), and these may be fed *Artemia*. Tadpoles should not be overfed, and uneaten food should be removed immediately to prevent contamination of the water.

Aquatic adult and larval salamanders feed on a wide variety of aquatic invertebrates, such as leeches, snails, crustaceans, and aquatic insect larvae; larger salamanders may also consume fish or other amphibians. In captivity, frozen brine shrimp, frozen whole minnows, and pieces of fish fillet have been fed successfully to several species of aquatic salamanders.

Terrestrial salamanders feed on earthworms, slugs, insect larvae and nymphs, and a wide variety of non-flying adult insects and other arthropods. If live prey is not available, chicken hearts are a reliable protein alternative, but they should be dusted with supplements (e.g., vitamins, calcium).

In captivity, *Ambystoma* and *Plethodon* species have been successfully maintained on earthworms and/or wax worms. *A. tigrinum* will feed on grain beetle larvae (*Tenebrio molitor*); however, it is preferable not to feed *Tenebrio* larvae as a long-term diet to amphibians because the heavy, chitinous exoskeletons are indigestible and may cause bowel obstructions. Amphibians fed only on Tenebrio larvae may develop nutritional deficiencies. Invertebrates that are recently molted are preferable as they are easier to digest.

6.4.1 *Xenopus*

Feeding regimes vary in the quantity, frequency, and type of food; however, a sufficient quantity and quality of food are required for optimal health (Reed, 2005). While frogs can survive long periods without food in the wild, there are physiological costs (Reed, 2005).

Insects are common prey for adult *Xenopus* (Reed, 2005); however, they will also accept non-living food items (Reed, 2005). Pellets of different sizes and protein concentrations are available, suited to the size and age of frogs and tadpoles (Green, 2010). They should be fed *Xenopus* pellets or a suitable alternative based on the ingredient list on the pellets' packaging. Beef liver is commonly provided; however, it has been associated with Chlamydia outbreak (Green, 2010) and may lead to disease if supplements are not given (Reed, 2005; Green, 2010). Maggots, crickets, and worms provide stimulation and enrichment for the frogs (Reed, 2005; Green, 2010). *Xenopus* develop food preferences, and therefore, when introducing a new food, it should be combined with the old food (Green, 2010).

Frogs may be fed at the end of the day to mimic their natural behaviour and eliminate disturbance during digestion (Reed, 2005). Feeding at this time may be particularly important for frogs that do not appear to be feeding. The feed should be consumed within 15-20 minutes of a feeding frenzy; the presence of feed in the water hours after feeding is an indication that too much was provided (Green, 2010). Uneaten food will contaminate the water, but removing it less than 3-5 hours after feeding may disturb the frogs and cause regurgitation (Reed, 2005). Therefore, the amount of feed should be carefully calculated to ensure the frogs get a sufficient amount and there is no residual food that would remain in the tank overnight.

At early stages of development, *Xenopus laevis* and *Xenopus tropicalis* are fed the same amount at a frequency of twice per day (McNamara et al., 2018). They should be fed algae until the tail begins to reabsorb. At 8 weeks of age for *X. laevis* and 5 months of age for *X. tropicalis*, the feeding schedule should be changed to once per day, five days a week (McNamara et al., 2018). At this stage, they can be fed an increasing quantity of food pellets as they grow. As adults, *X. laevis* is fed 2 times per week and *X. tropicalis* 3 times per week (McNamara et al., 2018).

6.4.2 Axolotls

Adult and juvenile axolotls can be fed a variety of foods, including frozen brine shrimp, salmon pellets, *Daphnia*, mosquito larvae, earthworms, frozen bloodworms, trout pellets, and beef liver or heart (Duhon, 2019). Adult axolotls are fed 3 times a week with 3-5 mm pellets (Khattak et al., 2014) to satiation and no more than five pellets at a time (Duhon, 2019). Pellets must sink to be consumed by axolotls. Uneaten food items should be removed a few hours after feeding (Khattak et al., 2014).

Axolotl larvae should be fed hatched brine shrimp (*Artemia*) and/or mosquito larvae. Mosquito larvae are readily produced and may be harvested at the developmental stage appropriate for the age of the axolotl larvae (NRC, 1974). Small *Daphnia* or very small worms (*Tubifex* sp. or *Enchytraeus* sp.) may also be fed.

When feeding pellets, the water should be changed daily for young animals and at least every other day for adults to prevent the accumulation of ammonia and other metabolic wastes and to prevent the establishment of large bacteria populations (Duhon, 2019).

6.5 ENVIRONMENTAL ENRICHMENT

Guideline 11

Environmental enrichment must be evaluated with respect to the particular species and life stage.

Animals should be free to engage in natural behaviours as much as possible. However, the specific requirements that allow natural behaviours depend on the particular animals, as amphibians occupy a large range of natural habitats and have varied life stages and forms of locomotion. In general, studies on environmental enrichment for amphibians are limited and have primarily focused on shelters for *Xenopus*, which appear to have both physiological and psychological benefits (Michaels et al., 2014a).

Important basic requirements that address the physical and behavioural needs of amphibians are discussed in other sections of this document:

- enclosures should be of sufficient size to allow amphibians to perform behaviours important to their welfare and promote the conduct of additional behaviours that will improve their quality of life (see Section 2.2, "Animal Enclosures in the Laboratory");
- shelters or other opportunities to hide should be provided (see Section 2.2.5, "Furnishings"); and
- amphibians should be size sorted and group-housed when beneficial to the animals (see Section 6.3, "Housing Management").

The addition of elements that may enrich the animals' environment must be considered in the context of the individual animal, the housing system, and the research requirements. Any structures added for enrichment should have smooth surfaces and rounded edges to reduce the risk of injury to the animals (Council of Europe, 2004, cited in Reed, 2005) and must not result in problems in water flow or cleaning that could be detrimental to the health of the animals.

Enclosures for frogs and toads must allow for natural activities such as jumping without risk of injury. Rubber mats can be added to the tank floor to prevent foot pad abrasions and pressure sores. Ramps and perches should be provided for variety. Boxes in which frogs can hide and places they can go to dry out when needed should also be provided.

Semi-terrestrial amphibian adults should be provided with access to both sufficient terrain to completely exit the water and sufficient water to submerge completely and swim, with a graded substrate that allows free movement in and out of the water. Salamanders should be provided with soil that has a gradient of moisture levels.

Burrowing species should be permitted to burrow and be fully covered to their preferred depth.

Arboreal species should be provided with a variety of complex structures of differing thickness, height, and density.

6.5.1 *Xenopus*

Facilities should strive to provide *Xenopus* with an enriched environment.

Floating objects on the water (e.g., commercially available plastic 'lily pads' or black bin liners cut into various floating shapes) provide *Xenopus* with cover from above and hiding places (Brown and Nixon, 2004, cited in Reed, 2005). *Xenopus tropicalis* have shown a preference for overhead cover in the form of both opaque, black covers and translucent, red covers; however, translucent covers allow for better observation of the animals by personnel (Cooke, 2018). The placement of these objects must enable the frogs to easily access the water surface to breathe (Reed, 2005).

Medium-to-large stones or rocks can add variety to a tank (Beck, 1994, cited in Reed, 2005), particularly if placed at a sufficient distance from the walls to allow frogs to pass through the space between the rock and the tank wall (Kaplan, 1993, cited in Reed, 2005). If there are concerns regarding sterilization or leaching, plastic molds of stones can be created from materials that have been determined to be safe (Reed, 2005).

Small stones should not be used as they can be ingested by the animals (Beck, 1994, cited in Reed, 2005; Chum et al., 2013) and may interfere with cleaning. In choice tests, frogs have not shown a preference for gravel flooring (Brown and Nixon, 2004, cited in Reed, 2005). The addition of plastic plants should also be evaluated with caution, particularly those with thin, pointed leaves or parts that can be easily pulled off.

6.5.2 Axolotls

Axolotls are solitary animals. Their basic needs of appropriate lighting and temperature and environments where vibration is eliminated or at least minimized are discussed in Section 3, "Facility Management and Personnel". However, there is a lack of information on additional elements that could provide enrichment for these animals. There is anecdotal evidence that axolotls may push around balls if they are provided, but the benefits have not been supported by scientific evidence. If gravel is used in the enclosure, it should be coarse to prevent axolotls from ingesting it (Duhon, 2019).

6.6 HUMAN CONTACT AND HANDLING

Handling should be kept to a minimum, and precautions should be taken to avoid stress or injury to the animals. The glandular skin and protective mucous layer of amphibians can be easily damaged by handling, and their lateral line system is highly sensitive (Elepfandt, 1996a, cited in Reed, 2005).

When handling amphibians, nitrile gloves should be worn and they should be wet, even when handling terrestrial amphibians (Sirois, 2015). Any traces of lotion or other products used by personnel, including soap residue, and the human skin's normal acidity, may be harmful to amphibians (Girling, 2013). Additionally, any roughness on the hands of personnel (i.e., nails and callouses) could cause abrasion of the amphibian's thin skin (Wright and Whitaker, 2001). Gloves also provide protection for personnel (see Section 13, "Human Safety").

Latex gloves should not be used, as they release inflammatory compounds that are harmful to amphibians. Gloves must also be free of powder.

There is concern that gloves may reduce the handler's sensitivity (Reed, 2005). Personnel handling amphibians must be aware of this and ensure excessive pressure is not applied to the animal.

Prior to handling amphibians, hands should be thoroughly cleaned, even though gloves are worn.

6.6.1 *Xenopus*

The preferred method of removing frogs from a tank is to use a small container to lift them out, along with some of the tank water. This will likely result in less stress or harm for the animal than physically handling them; however, it may not be practical in tanks with a large number of frogs, in deep water, or where specific frogs need to be captured (Reed, 2005). Fine nylon nets are considered to cause less damage to the mucous layer and are less traumatic than handling (Green, 2010); however, some caution that nets may cause micro-lesions on the frog's skin or the frog's digits may become trapped (Reed, 2005). Nets should be cleaned between uses (see Section 6.7, "Cleaning and Sanitation").

When handling frogs is necessary, there are two common methods for holding *Xenopus*: 1) placing the animal's head between the handler's first two fingers, with the animal's body across the palm of the hand and the neck gently restrained by the handler's thumb; or 2) placing one of the handler's hands across the animal's back, with a forefinger between the animal's hind legs and wrapping the rest of the hand around the animal's middle (Reed, 2005). If other methods are to be used, they should be verified with the veterinarian.

6.6.2 Axolotls

Fine nylon nets should be used to handle axolotls. Axolotls are slippery to hold, and there is the possibility of squeezing them and causing harm.

6.7 CLEANING AND SANITATION

Cleaning practices should be based on the tank or system design, the feeding regime, and the quality of water entering the system (Reed, 2005).

For aquatic species, consideration must be given to maintaining the water quality while minimizing disturbance to the animals (Reed, 2005). Frogs that were subjected to daily water changes have been shown to have a slower growth rate than those in a tank where water was only replaced weekly (Hilken et al., 1995, cited in Reed, 2005). Complete water changes should be avoided. Only one-quarter to one-half of the water should be siphoned from the bottom of the tank, along with food detritus and feces, and replaced by clean water of the same temperature. When complete water changes are necessary, they should be done at the same time of day (Schultz and Dawson, 2003) and preferably post-feeding when the water quality is poor (DeNardo, 1995, cited in Reed, 2005). It is important that frogs are held in water of the same temperature and physicochemical properties (i.e., salinity, pH, etc.) while their enclosure is being cleaned.

For terrestrial animals, such as salamanders, there should be a daily check for animal waste (fecal pellets are typically visible), and the waste should be removed with minimal change to the substrate. Significant substrate change should occur annually. When there is a significant soil change, the balance of beneficial and undesirable flora and fauna in the soil may be disrupted, and blooms of fungus or mould often occur. When large changes of the substrate are necessary, some of the original soil should be maintained to sufficiently seed the new substrate with beneficial flora and fauna. The pH level should also be maintained (Sugalski and Claussen, 1997; Mushinsky, 1975).

For most situations, furnishings and enclosures should be cleaned with hot water alone. Tanks and equipment (e.g., nets and buckets) can be cleaned in hot water using a soft brush or sponge (Green, 2010). Where there are disease concerns, more stringent practices may be required, followed by an acid wash.

Detergents and disinfectants should be avoided as they are harmful to amphibians. When disinfectants are used, the surfaces must be thoroughly rinsed and dried and must not pose a risk to the animals. If sterilizing agents are necessary, food-grade hypochlorite should be used and rinsed thoroughly several times, and tanks should be dried before amphibians are returned (Reed, 2005). Soaps should not be used as they have residues that are hard to remove. Any chemicals used for cleaning (e.g., detergents, oxidizers) must not come into direct contact with the animals.

If plastic containers are used, they must be kept very clean as bacteria can grow along the bottom and sides.

6.8 RECORD KEEPING

General record-keeping requirements are detailed in the *CCAC guidelines: Husbandry of animals in science*, Section 12, “Record Keeping” (CCAC, 2017). As noted above in Section 6.2, “Animal Observation”, systems supporting amphibians must be checked daily and the animals must be observed regularly at a frequency described in an SOP for each species. Records must be kept of these checks and observations.

7 HANDLING AND RESTRAINT

7.1 HANDLING

Guideline 12

Amphibians should only be handled when necessary, and the handling time should be minimized.

For general handling for husbandry activities, when necessary, see Section 6.6, "Human Contact and Handling".

7.2 PHYSICAL RESTRAINT

The method of restraint will depend on the body shape of the animal (Girling, 2013). Some amphibians do not tolerate physical restraint well (such as small plethodontids, which easily overheat in the hand), and attempts to restrain these species could cause injury to the animals.

Medium and large frogs and toads (approximately 5 g and larger) should be grasped around the waist (immediately anterior to the hind limbs) with the hind limbs fully extended. During restraint, the animals should not be allowed to flex their hip and knee joints to prevent kicking. For larger animals, a second grip should be maintained around the forelegs. Girling (2013) suggests cupping one hand around the pectoral girdle immediately behind the front limbs with the other hand under the hind limbs. Other techniques for restraining frogs for injections are described by Browne (n.d.).

When restraining small salamanders, an open flat hand should be used to apply even pressure over the animal's entire body. Pressure on the tail should be avoided. Medium and large salamanders (approximately 5 g and larger) should be grasped in the middle of the body between the forelimbs and hind limbs. Girling (2013) suggests holding the pectoral girdle from the dorsal side, with the index finger and thumb around one forelimb and second and third fingers around the other, and the opposite hand holding the animal in a similar manner.

Salamanders must not be grasped or picked up by the tail, as tail autotomy can occur. Rough handling can also cause some salamanders to shed their tails (Girling, 2013). Although this is not a serious injury, it may influence future growth and reproduction by depriving the animal of fat stores and affect the behaviour of the animal, as has been reported in reptiles (see Ballinger, 1973; Derickson, 1976; Bellairs and Bryant, 1985).

Larval and neotenic salamanders must not be grasped around the head or neck, as this can damage their gills.

7.3 CHEMICAL RESTRAINT

Chemical restraint may be necessary for animals that are very small or otherwise difficult to handle for a particular procedure or where there is the potential for physical restraint to cause injury or excessive stress for the animal. A veterinarian experienced with using anesthetics on amphibians should be consulted when planning to use chemical restraint in a new project or a new species. Different species and even different individuals may respond differently to anesthetics. Published literature and people experienced with the species should be consulted. For information on the use of anesthetics, see Section 10.10.1, "Anesthesia".

8 HEALTH AND DISEASE CONTROL

Guideline 13

All amphibians should be included in an animal health program regardless of whether they are housed in the main animal facility or at another location within the institution.

Veterinary professionals must be engaged in the development of the health program, which should be approved by the animal care committee and overseen by people competent in evaluating the health of amphibians. The animal health program should include:

- strategies to prevent conditions that may contribute to ill health, which are suited to the health status of the animals and the intended studies;
- systematic evaluation of individual animals and the health status of each colony to monitor animal health and detect latent disease; and
- an emergency plan for the management of disease in the event of a suspected outbreak.

8.1 DISEASE PREVENTION

Guideline 14

Strategic measures for disease prevention should include a program for disease control and a system of regular monitoring and reporting for health assessment purposes.

Animals should be free of pathogens and clinical diseases unless they are part of an approved protocol. A veterinarian should be responsible for the development of SOPs to limit the risk of introducing a disease into the facility, and should be available for consultation on all matters relating to the health of the animals.

The disease prevention and control plan should address the following:

- procurement – amphibians coming from a supplier should have a recent satisfactory health report provided by the supplier and undergo a thorough health assessment upon arrival (see Section 4, "Procurement");
- quarantine – newly arrived animals should be kept separate from other animals in the facility (see Section 4.5, "Reception of Amphibians at an Institution");
- facilities and their management – facilities, equipment and management practices should be in place to prevent airborne, waterborne, direct contact or fomite transmission of microorganisms, water contamination, pest infestations, and contaminants from external sources;

- husbandry – amphibians should be fed a high-quality diet, and practices should be in place for effective sanitation and prevention of overcrowding;
- biosecurity for the animals – SOPs should limit access to the animal facilities;
- temporary holding – plans should be in place for holding contaminated animals separate from other animals in the facility in the event of a disease outbreak and should include a disease prevention strategy.

It is important that all of these components are included in the disease prevention and control plan. As noted by Green (2010), most of the clinical problems seen in *Xenopus* can be attributed to husbandry, handling, and management practices.

A routine and comprehensive health monitoring program is essential; however, due to limitations associated with testing, it will not be sufficient to ensure the animals are free of pathogens or infectious agents that may affect research (i.e., testing is always retrospective). As infectious agents may affect experimental data without causing disease, biosecurity for the animals is an important component of a preventative health program.

8.2 HEALTH MONITORING AND DISEASE DETECTION

Guideline 15

Standard operating procedures should be developed for assessing animal health, providing health care, and treating common health problems for the animals; these should be reassessed every three years or sooner to ensure relevance.

SOPs should be developed for routine health checks and welfare assessment for individual animals and each colony, based on the species, sex, life stage, age, and health status of the animals, the housing system, the type of research, and the potential effects on other animals in the facility. Animal monitoring requirements for health and disease control will also depend on the length of time the animals are housed. Health monitoring programs may include the use of environmental monitoring of both the room (e.g., temperature and humidity) and the enclosure (e.g., water quality parameters, as described in Section 3.1.3, “Air and Water Quality”, or monitoring the substrate or water column for fungus or mold). Evaluation procedures need to be determined (e.g., test intervals, selection of agents, and verification). It is important that testing methods and samples are specific to the disease of interest and, where possible, adhere to the Three Rs principle of reduction.

Health monitoring and disease detection programs should be updated over time in response to the regional prevalence of diseases in the area and new information on the health of amphibians. The literature should be reviewed for information on diseases affecting the species and procedures for their detection. In testing for pathogens, the use of molecular assays of samples taken from the animals and/or their environment is strongly encouraged where applicable, as they can replace sentinel animals and result in a reduction in the numbers of animals maintained in the facility.

There should be procedures in place to ensure any animal health concerns or other potential animal welfare issues are documented and communicated to the veterinarian in a timely manner. Documentation should include actions taken, outcomes resulting from the actions, and long-term improvements.

Signs of illness in amphibians are rarely specific to a particular disease. Diagnostic testing and treatment for diseases in amphibians is discussed by Wright and Whitaker (2001) and Gentz (2007). General signs of illness in *Xenopus* are described by Green (2010).

8.2.1 Common Diseases and Conditions

Many infections, such as Red Leg (a bacterial infection in frogs) and *Saprolegnia* (a fungal infection in salamanders), originate through abrasions or wounds to the skin. Other contributing factors include stress from unsanitary conditions, prolonged exposure to cold, and overcrowding (Reed, 2005).

Red Leg is a common disease presentation that can be caused by various agents, including Aeromonas. Red Leg can spread through a colony, and potentially infected animals must be isolated (Reed, 2005). A variety of signs of Red Leg have been described, including cutaneous ulcers and lesions, cutaneous hemorrhages, lethargy, emaciation, swelling, coelomic effusions, sloughing, necrosis, and trembling (Densmore and Green, 2007; Reed, 2005). However, it may also result in death with no visible signs (Densmore and Green, 2007; Reed, 2005). Necropsy may be unreliable for diagnosis if performed more than 1-3 hours after death, as bacteria rapidly invade amphibian tissues (Densmore and Green, 2007). Treatment should be provided in consultation with the veterinarian. Suggested treatments are described by Densmore and Green (2007) and Reed (2005).

Superficial fungal infections, such as *Saprolegnia*, can start in minor abrasions and appear as an opaque, usually fuzzy, white area on the skin. They are of particular concern for larval salamanders that are housed together, as they nip at one another. The appearance of fungal infections, especially among larval salamanders, is an indicator of attempted cannibalism, and the animals should be separated.

Ranavirus is present worldwide and is responsible for massive mortality events for many amphibian species (CWHC, 2020; ACVP, 2020). There can be no clinical signs of ranavirus infection, or the signs can resemble bacterial sepsis. Bacteria are often present as secondary pathogens. Ranavirus can be devastating for amphibians, but good quarantine and husbandry practices should help prevent outbreaks in research facilities. For more information, see CWHC (2020) and ACVP (2020).

Other diseases that should be considered in a health screening program include:

- chytridiomycosis, which is caused by a fungal pathogen that has significantly contributed to the decline in wild amphibian populations and has been identified as a problem in captive amphibians;
- mycobacteriosis, a bacterial disease that commonly affects amphibians;
- chlamydiosis, which is common among anurans; and
- nematodes, including *Pseudocapillaroides xenopi*, which affects *Xenopus* (Densmore and Green, 2007), and *Rhabdias*, which affects wild caught frogs and salamanders (Fox et al., 2005).

The development of spontaneous tumors in amphibians should also be considered during monitoring, as they have been reported for many years (e.g., Balls, 1962) and continue to occur (Tsonis and Del Rio-Tsonis, 1988; O'Brien et al., 2017).

In *Xenopus*, bloating disease (also referred to as dropsy) is an osmoregulatory problem resulting in accumulation of fluid in the subcutaneous space (Green, 2010). It often occurs in association with infections, renal disease (Green, 2010), sepsis, and cardiovascular and lymphatic diseases. Edema (or bloat) has been observed in frogs held in water with low conductivity, and this may be resolved through the maintenance of proper salinity/conductivity levels (V. Langlois, personal communication, 2020). Whenever symptoms of bloat are observed, water conductivity/salinity should be investigated, and testing for bacterial disease should be considered in consultation with the veterinarian.

Food toxicity is associated with uneaten, partially decomposed food that contributes to the growth of fungal hyphae. Once symptoms appear in animals, death will follow within a few hours. The least affected animals may be saved by moving them to a separate, clean tank with a high water flow and low turbulence.

Diseases may also originate from housing and husbandry practices, such as gas bubble disease from supersaturated water, nutritional diseases involving insufficient or excessive intake of calories or particular vitamins and minerals (Densmore and Green, 2007), dehydration, and thermic shock.

For information on these and other common diseases affecting amphibians, see Densmore and Green (2007) and Reed (2005).

8.3 DISEASE MANAGEMENT IN THE EVENT OF AN INFECTIOUS OUTBREAK

Guideline 16

A management plan must be in place to deal with unanticipated disease outbreaks.

A management plan must be developed to deal with disease outbreaks within the facility and from outside sources and to prevent pathogen transmission and infection recurrence. Plans should include a communication strategy involving veterinarians, veterinary and animal care personnel, investigators, the facility manager, and the animal care committee. Access to quarantine facilities or a means of isolating the animals must be available. Arrangements should also be made with an animal health laboratory to determine the best procedures for submitting samples and specimens for necropsy, bacteriology, and fungal culture.

For infectious disease outbreaks, the veterinarian must be consulted to ensure that the techniques employed will eradicate the pathogens. Typical procedures may include quarantining the room in which the disease is discovered and tracking and testing any animals that were recently moved from that "source" room. Follow-up actions (e.g., treatment or depopulation) will depend on the nature and extent of the outbreak, the health status of the animals, and the type of research. If infected animals are to be euthanized, proper containment measures must be in place for handling and disposing of the animals and the contents of their enclosures and decontamination of the enclosures and room to prevent the spread of disease.

9 WELFARE ASSESSMENT

General guiding principles for welfare assessment of all animals used for scientific purposes are described in the *CCAC guidelines: Animal welfare assessment* (CCAC, 2021). Information in this section builds on the general guidelines by focusing on indicators for assessing the welfare of amphibians, keeping in mind that any indicator must be tailored to the species and life stage of the animal of interest.

Guideline 17

All amphibians maintained in an institution must be subject to routine welfare assessments.

An animal welfare assessment plan should include the use of observations and other tools that collectively provide information on the health, behaviour, and physiology of the animal. As noted in the *CCAC guidelines: Animal welfare assessment* (CCAC, 2021), information should be obtained through a mixture of animal-based measures, resource-based measures, and management-based measures. Welfare assessments should involve protocol authors and their delegates, veterinarians, and animal care personnel, and where possible, draw on information gathered through research, veterinary, and husbandry activities.

Animal-based measures include observation of the animal within the enclosure (see Section 6.2, "Animal Observation"), health assessment upon receipt of the animal at the institution (see Section 4.5, "Receiving Animals") and as part of the animal health program (see Section 8, "Health and Disease Control"), and any additional information on the health, behaviour, or physiology of the animal obtained through the conduct of experimental procedures. While it is important that amphibians are observed regularly, some animal-based measures can cause disturbance (e.g., for species that hide, digging the animals up or removing their cover to facilitate observation). Thus, the frequency of such disturbance must be carefully considered when designing the welfare assessment plan. Combining observation of the animal with resource-based measures and management-based measures can help minimize disturbance while obtaining the necessary information to assess their welfare. Generally, animal-based measures are the best for identifying the actual welfare status of the animals but are less useful in identifying specific causes of poor welfare.

Resource-based measures evaluate the suitability of the enclosure for the particular animal being housed. Michaels et al. (2014b) describe key elements of the natural habitat of amphibians that should be assessed in the captive environment. Criteria for the development of resource-based measures are described in Section 2.2, "Enclosures" (e.g., enclosure size and design), Section 3, "Facility Management and Personnel" (e.g., water quality, lighting, and temperature), and Section 6, "Husbandry" (e.g., group-housing requirements, nutrition, and environmental enrichment). Resource-based measures are most useful for identifying potential causes of poor welfare.

Management-based measures focus on the assessment of records (husbandry records, medical records, mortality/morbidity records, experimental records, etc.) to identify potential sources of welfare impacts to animals. Similar to resource-based measures, management-based factors are useful for identifying potential causes of poor welfare. They are particularly useful in tracking these potential causes over time.

9.1 WELFARE INDICATORS

Amphibians include a wide range of species, which collectively occupy very diverse habitats in nature (i.e., from tropical regions to temperate climates with cold winters). Determination of suitable welfare indicators must take both of those factors into consideration.

It is important for investigators to have a good understanding of the biology and behaviour of the species that they are working with in order to make appropriate assessments of their welfare. It is also important to recognize how different factors could affect behaviour and physiological parameters in different species.

Changes in behaviour or unexpected behaviours warrant further investigation into environmental conditions to assess their relevance to the welfare of the animal. Behavioural welfare indicators often fall within a spectrum and cannot be evaluated by a simple checklist. Additionally, they must be considered within the context of the animal's environment, as a particular change in behaviour may be indicative of varying stress levels, depending on the situation. For example, a diminished avoidance response could indicate lethargy, or it could mean that the frog is habituated to personnel. As well, activity levels can fluctuate in response to factors that do not necessarily relate to the welfare of an individual (e.g., seasonal changes).

In general, the following behaviours and physiological parameters could be used to make welfare assessments on individuals or group-housed amphibians:

- behavioural indicators of welfare
    - changes to feeding behaviour (e.g., time to feed and amount of food consumed in relation to pre/post procedure or during acclimation or feed transitions)
    - unexpected behavioural response
        - requires knowing what behaviours are 'expected' under typical captive settings (e.g., healthy individuals should exhibit a startle response when their environment is suddenly disturbed, whereas they should be less reactive (i.e., no startle response should be seen) to daily routine husbandry practices such as feeding)
    - social interactions (e.g., high amounts of aggression being exhibited within a colony)
- physiological indicators of welfare
    - corticosterone levels
        - some studies have looked at corticosterone in relation to bacterial infection susceptibility, others use corticosterone as a measure of stress (Ohmer et al., 2017, 2015; Gabor et al., 2015; Cikanek et al., 2014)
    - growth and reproduction
        - increased stress hormone is linked to reproductive decline and early metamorphosis of larvae (Michaels et al., 2014b)
    - colour and appearance of the skin
    - departure from normal appearance of fecal pellets for frogs and salamanders (e.g., change in colour or form)

Investigators should consult the literature and use the least invasive indicators that are appropriate for the particular animals and research. For example, the use of skin swabs to measure stress hormones (Santymire, 2018) may be appropriate for some situations.

9.1.1 *Xenopus*

Behaviours typically seen in *Xenopus* in a captive setting are described by Holmes et al. (2016) and Chum et al. (2013). Holmes et al. (2016) provide an ethogram for recording *Xenopus laevis* behaviour, which includes both common behaviours related to movement and short-duration behaviours related to breathing, sloughing of skin, and movement in relation to the tank wall. Chum et al. (2013) provide a detailed description of the appearance and activity level of healthy *Xenopus* versus animals that are experiencing stress or ill health.

Examples of deviations from normal behaviour that may warrant investigation include sudden changes in activity level (e.g., lethargy or jumpy), failure to feed properly, and diminished righting reflexes (Reed, 2005). In terms of appearance, *Xenopus* normally have moderately slimy skin with a glossy appearance (Sive et al., 2000 cited in Reed, 2005; Tinsley, 2010) and changes such as the appearance of dry, flaking skin, excessively slimy skin, or dull skin with subcutaneous hemorrhaging require attention (Reed, 2005; Tinsley, 2010). Skin sloughing is normal and plays a role in regulating infection in *Xenopus*; however, high quantities of sloughed skin in the tank (Tinsley, 2010) or change in the rate of skin sloughing (Ohmer et al., 2017) could be indicative of welfare concerns.

9.1.2 Axolotls

Skin colour and the shape of the dorsal fin and tail are important welfare indicators. Skin that appears faded from its original colour (i.e., as if there was a film over it) and patchy and/or a curled dorsal fin and tail indicate poor health.

10 EXPERIMENTAL PROCEDURES

Guideline 18

The least invasive method suited to the goals of the study must be used with consideration of the potential impacts of the procedures on the amphibians and measures to reduce those impacts.

As with other species, procedures should not be conducted in animal rooms unless they are very minor, and measures should be taken to reduce potential impacts that the procedures may have on other amphibians. There is some evidence that amphibians may be affected by the production of alarm pheromones (e.g., Fraker et al., 2009).

For some types of research, it is important to alter the timing of metamorphosis, and this will involve altering the environmental conditions from those described in earlier sections of this document.

The institutional animal care committee must review all protocols involving experimental procedures (see CALAM, 2020). For routine procedures, SOPs approved by the animal care committee should be available to all personnel involved with the animals to ensure consistency of procedures and animal care. Where new procedures are proposed, SOPs should be developed in consultation with an expert in the subject matter, and input should be sought from stakeholders (i.e., investigators, safety officers, animal care personnel) before they are approved and implemented. SOPs should be reviewed regularly and updated as new information becomes available (CCAC, 2006). All procedures should be documented, and records should be kept in electronic files or in close proximity to the housing or procedure areas and be accessible to the veterinary team, animal care committee, and the research team.

Institutions should have a policy or SOP on repeated procedures on all animals. The frequency, duration of intervals between procedures, and the total number of procedures that may be performed on the same animal during its lifetime must be considered. The SOP must take into account the invasiveness, pain, and distress associated with those procedures and their impact on the welfare of the amphibian, both in the short and long term (CCAC, 1998).

Procedures that adversely impact animals should be avoided where alternative methods are effective in achieving the study outcomes.

All procedures could have the potential to cause pain and distress. Many seemingly routine procedures are more complicated when conducted on amphibians because of their small size. Procedures must be performed by competent people that have been properly trained by personnel with appropriate expertise (for details, see the *CCAC guidelines on: training of personnel working with animals in science* (CCAC, 2015)). Where possible, it is preferable to use the expertise of a veterinarian and experienced animal care personnel to carry out procedures.

As techniques advance, refinements will continue to evolve in many areas, and investigators, veterinarians, and animal care committees should evaluate new evidence on refinements and consider their implementation.

> **Guideline 19**
>
> Endpoints must be developed and approved by the animal care committee prior to the commencement of the study to minimize any negative impacts of the procedures on the animal.

The *CCAC guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (CCAC, 1998) provides the following definition of an endpoint: "the point at which an experimental animal's pain and/or distress is terminated, minimized or reduced, by taking actions such as killing the animal humanely, terminating a painful procedure, or giving treatment to relieve pain and/or distress." Investigators, in consultation with the veterinarian, must establish appropriate and study-specific endpoints (e.g., initiation of treatment, termination of a procedure, and euthanasia) and plans for monitoring. Key references relevant to the particular study should be consulted in determining the earliest practical endpoints.

Defining endpoints can be challenging, as amphibians generally do not display the range of clinical signs found in other laboratory animals, and it may be difficult to interpret the severity of a particular condition when signs are presented (Alworth and Harvey, 2007). Welfare indicators, such as inappetence, changes in the skin, fluid retention, changes in body weight, loss of ambulatory function, and levels of stress hormones (see Section 9, "Welfare Assessment"), can provide a basis for defining endpoints. However, given that many amphibians do not eat on a regular basis and are normally not very mobile, indicators linked to feeding behaviours and movement are often difficult to assess (Alworth and Harvey, 2007).

Potential endpoints for toxicity studies involving larval amphibians, and *Xenopus laevis* in particular, are described in OECD test guidelines (OECD, 2014).

Where an animal model may be in development or new to an investigator, pilot studies should be performed to establish endpoints.

Appropriate monitoring frequency must be established based on the level of invasiveness of the protocol, expected clinical or other signs, the progression of the animal's condition, the particular animal model, and the individual animal (e.g., previous experience). Monitoring should be documented.

Monitoring for endpoints should be a cooperative effort involving investigators, veterinarians, and veterinary and animal care personnel. Where appropriate and in accordance with the level of invasiveness of the protocol, monitoring score sheets incorporating several parameters of assessment can be helpful in monitoring for endpoints. When assessing the behaviour of an animal, it is important that personnel be familiar with normal or expected behaviour so that they can recognize deviations.

Animals experiencing nociception that cannot be relieved and that is not approved as part of the research protocol must be euthanized promptly.

10.1 ANIMAL MODELS

Amphibians are used as animal models for a wide range of studies in physiology (Burggren and Warburton, 2007) and ecological research (Hopkins, 2007).

Investigators or study directors should decide whether amphibians are required for the study and if so, which species, strain(s), and life stage provide the best model of the biological processes involved in their work, taking into account the special needs of the species/strain/life stage, the ethical or welfare considerations of working with those animals for a given experiment or study, and their availability. Animals that have special requirements must not be obtained until measures are in place to care for them appropriately. Particular studies may need to be redesigned if those requirements could pose difficulty with maintaining the health and welfare of the animals or be intensified as a result of the experimental interventions. The measures required in these situations may include special or additional technical expertise and highly trained personnel.

10.2 ADMINISTRATION OF SUBSTANCES

The route of administration depends on the type of substance (e.g., whether it is water-soluble). For aquatic species, immersion should be used in preference to other routes if appropriate for the substance being administered. Some substances, such as peptide hormones, cannot be administered through immersion and require injection. When injection is required, the species and age of the animal and the particular substance must be taken into consideration.

Due to the permeability of their skin (Kaufmann and Dohmen, 2016), immersion can be used on terrestrial species and can target a specific area of the body, such as a limb. For terrestrial species, injection into the dorsal lymph sacs is also appropriate for some substances.

10.2.1 *Xenopus*

Substances injected into the dorsal lymph sac are usually rapidly absorbed into the blood (Reed, 2005); however, this can be influenced by the type of substance. For example, lipid-soluble substances may be less effective due to the high lipid content of lymph. Substances can also be injected intramuscularly into the thigh (Wolfensohn and Lloyd, 2003, cited in Reed, 2005; Green, 2010) or intraperitoneally (intracoelomically) while the frog is placed on its back (Wolfensohn and Lloyd, 2003, cited in Reed, 2005; Green, 2010). Information on appropriate needle gauge and volumes is provided by Green (2010).

10.2.2 Axolotls

Immersion is often used for administration of small molecules to study signaling pathways. Substances can also be injected subcutaneously, intraperitoneally, or intramuscularly.

10.3 COLLECTION OF BODY FLUIDS OR TISSUE

10.3.1 Blood Collection

Collecting blood from amphibians is challenging due to their small size and the difficulty in accessing their blood vessels (Reed, 2005). Anesthetics should be used as appropriate to restrain the animal and limit stress, and methods for proper visualization of blood vessels may be needed (Heatly and Johnson, 2009). The selection of appropriate-sized needles is important to minimize tissue trauma (Heatly and Johnson, 2009).

Common sites for blood collection in amphibians include the ventral caudal tail vein and the ventral abdominal vein; cardiac puncture is also used but is associated with greater risk to the animal, requires anesthesia (Heatly and Johnson, 2009), and should be a terminal procedure. Blood collection from the facial (maxillary)/musculo-cutaneous vein of species of the family Ranidae has been described as minimally invasive, but care must be taken to ensure the eye or tympanum are not lacerated (Forzán et al., 2012). The ventral tail vein is the preferred site for collecting blood from salamanders (Gentz, 2007).

10.3.1.1 Survival Blood Collection

For healthy animals, up to 1% of their body weight (or 5-10% total blood volume (Heatly and Johnson, 2009)) can be collected. For amphibians that are not in good health, blood collection should be limited to half of that amount (Wright 2001a, cited in Gentz, 2007).

There is limited information available regarding repeat blood collection in amphibians, and therefore it should not be performed unless absolutely necessary for the study and approved by the animal care committee. If serial draws are necessary, different sample sites should be used, and the time between draws should allow for replacement of the blood volume. The animals must be monitored for changes in behaviour (listlessness), hydration, and colour change lasting longer than 1 hour, which could indicate that too much blood has been taken. If blood loss is too great, there is potential for hypovolemic shock, resulting in increased heart rate, dry mucous membranes, lethargy, and eventually death by multiple organ failure.

10.3.1.1.1 Xenopus

Common blood collection sites in other amphibians, such as the tail vein, lingual vein, femoral vein, and abdominal vein, are inaccessible in *Xenopus* (Green, 2010). Clipping the toe web can provide a few drops to 0.5 ml of blood; however, this procedure has the potential to cause pain and distress, and a local anesthetic should be used (Wolfensohn and Lloyd, 2003, cited in Reed, 2005). Amputation of a digit should not be performed as it has the potential to cause pain and result in infection, and the sample will be contaminated with lymph and tissue (Green, 2010). Therefore, the most reliable technique for blood collection is commonly a terminal procedure performed under anesthesia (Green, 2010).

10.3.1.2 Terminal Blood Collection

Cardiac puncture under general anesthesia can be used as a terminal procedure and should be followed by a secondary method (e.g., an overdose of an anesthetic or a physical method) prior to recovery to ensure death (Reed, 2005).

10.3.2 Urine and Feces

Narayan et al. (2013) describe a method of urine collection in frogs that involves gently holding the frog above a sterile cup and briefly massaging its abdomen; urination should occur within one minute. Wright and Whitaker (2001) also provide information on urine collection.

Fecal samples are commonly collected by feeding frogs and placing them in plastic containers lined with paper towel, and allowing sufficient time for the frogs to defecate (Whitaker, 2016).

Cloacal wash, described by Wright and Whitaker (2001), is commonly performed to collect fresh fecal samples for microscopic analysis. This involves placing a well-lubricated rubber tube of appropriate size into the cloaca and using a 1.0 ml syringe attached to the other end of the tube to infuse a small amount of saline, which is aspirated back out (Nugent-Deal, 2011).

10.4 IMPLANTS

For amphibians, implants include transponders (microchips) or visible implant elastomer (VIE) tags for identification. They also include materials implanted for other purposes, such as gelatin beads soaked with specific proteins that are grafted in some animals to study tissue regeneration (e.g., Satoh et al., 2015).

There is evidence of migration of some implanted devices within frogs, followed by elimination through the bladder (Divers and Stahl, 2019; Borrell, 2010). In addition, passive integrated transponders (PIT) have been shown to disrupt the protective bacteria on the skin of frogs for two weeks following insertion, making the animals more susceptible to infection (Antwis et al., 2014a); therefore, VIE tags may be a better option for identification (Antwis et al., 2014b).

10.5 PROCEDURES FOR GENETICALLY MODIFIED AMPHIBIANS

The selection of methods to generate new genetically modified strains should be made with consideration of the Three Rs; for example, reducing the number of animals used in creating and maintaining each line (some methods are more efficient than others) and considering the overall welfare impacts. Procedures for the generation of genetically modified animals should be reviewed by the animal care committee during protocol review, in keeping with the rapidly evolving nature of genetic modification and advances in research on animal welfare. Submission of protocols for renewal by the animal care committee should include a report from the investigator on the efficiency of the methods used to produce new strains, which may include reference to published papers.

Successful archiving technologies contribute to reduction and refinement. Efficiency in these technologies can vary among species, and expertise in this area should be sought in the development of a new line.

Amphibians to be involved in procedures for genetic modification should be in good health and exhibit normal behaviour. For resources providing detailed information on genetic modification in amphibians, see Appendix 1.

10.5.1 Collecting Samples for Genotyping

The sampling method should be the least invasive method that can provide the quantity and quality of tissue required for the particular genotyping method. Where possible, swabs should be used to provide samples for PCR testing. The use of more invasive methods that involve the removal of tissue from the tail or digits must be justified in the animal use protocol.

10.5.2 Phenotyping

Some procedures that are acceptable for animals that have not undergone genetic modification may not be acceptable for genetically modified amphibians with altered phenotypes. Procedures may need to be modified or avoided when the animals' ability to respond to stress is compromised. This includes the choice of procedures for phenotyping.

Once the animals are phenotyped, any additional information related to animal welfare should be given to the animal care committee as soon as possible. Stable germ-line transmission does not necessarily mean that there is a stable phenotype or stable animal welfare, since phenotypes can change (e.g., be age-dependent, have background effects, require homozygosity, or require breeding to other mutant lines). Appropriate monitoring is needed for the life span of the animal or when the genetic background is changed.

Investigators should take reasonable steps to publicize to the research community all available phenotypic and welfare information, along with strategies for mitigating problems with genetically modified strains.

Genetically modified amphibians may respond differently to drugs and feed and to a number of experimental conditions, compared to animals of the same species that have not undergone genetic modification. These changes in response may be the result of differences in the animal's metabolism and are particularly relevant to the use of anesthetics and to the use of the animals for testing new drugs or for toxicity studies.

10.6 IMAGING

Imaging plans must be developed in consultation with a veterinarian. Although studies involving repeated imaging can reduce the number of animals required for a research study, the procedures create numerous occasions for animals to be stressed. Other factors to consider include repeated injections, anesthesia, handling, transportation, experimental conditions (e.g., tumour burden or surgery), and fasting (Hildebrandt et al., 2008). Aquatic amphibians should be well hydrated during the procedure. All of these factors should be addressed in relation to both the welfare of the animals and the validity of the imaging results.

Given the significant impact of repeated anesthesia on the physiology of an animal, consideration should be given to the number of times and frequency of imaging. For serial imagery, it is important that animals are monitored between imaging sessions.

The schedule for imaging should be developed based on the anticipated outcomes of an intervention or timeline of an age-dependent change and the welfare of the animals.

Equipment should be thoroughly cleaned, disinfected, rinsed, and dried between uses to minimize the possibility of cross-contamination, especially when sharing equipment.

10.7 BEHAVIOURAL STUDIES

Behavioural studies on amphibians include mating and courtship, social behaviour, feeding behaviour, environmental preference (e.g., background tank colour), and predator/prey behaviour (primarily involving larvae). A healthy animal with a good welfare status that is well acclimated to the housing environment is critical to achieving a valid and interpretable outcome of any behavioural testing regime.

Where possible, a reward strategy (e.g., highly preferred food) should be used to motivate an animal rather than using aversion. Aversive stimulation, deprivation, or restriction of resources should only be used when there is no alternative. Motivational studies using shock, aversion stimuli, and/or food restriction should be justified to the animal care committee and used in the least invasive fashion and for the shortest duration possible.

Equipment should be thoroughly cleaned, disinfected, rinsed, and dried between uses to minimize the possibility of cross-contamination, especially when sharing equipment.

10.8 FOOD AND FLUID INTAKE REGULATION

Studies involving food or fluid intake regulation require the establishment of endpoints (e.g., for skin condition) and close monitoring of the animals. Knowledge of the individual animals is critical, as there is a large spectrum of requirements within amphibians in terms of hydration, feeding, and digestion. The digestive system of amphibians is considerably different from warm-blooded animals, and they do not eat as regularly. Amphibians on food restriction diets should be carefully monitored for any cachexia regarding weight and body condition. If the weight change of an adult animal approaches 10%, the frequency of monitoring should be increased.

10.9 COLLECTION OF OOCYTES

Animals must be in good health before planning oocyte collection, and there must be sufficient time between collections to allow the animal to recover. Institutions should set limits on the number of surgeries on each animal, based on the health of the animal and the quality of the eggs.

The general precautions for surgery apply, including anesthesia and analgesia (see Section 10.11, "Surgery"). During the week following surgery, the animals should be monitored for evidence of excessive inflammation of the incision site, suture dehiscence, or behavioural abnormalities indicative of illness (e.g., change in appetite, listlessness, or lethargy).

10.10 ANESTHESIA AND ANALGESIA

10.10.1 Anesthesia

Guideline 20

Anesthetics must be used in procedures where there is expected to be noxious stimuli and in experiments entailing extensive handling or manipulation with a reasonable expectation of trauma and physiological insult to the animal.

Anesthesia is generally defined as a state caused by an applied external agent, resulting in depression of the nervous system, leading to loss of sensation and motor function. As with other animals, amphibians require appropriate anesthesia when undergoing or when exposed to potentially painful procedures (Mitchell, 2009). Anesthesia is also needed for some procedures involving handling to reduce stress and minimize the risk of injury due to escape behaviours.

Guideline 21

Amphibians must be continually monitored during anesthesia, from induction to recovery.

Care should be taken to maintain the animal at its preferred body temperature during induction and recovery. The length of time required for induction and recovery from anesthesia depends on the species, life stage, anesthetic, temperature, and depth of anesthesia. Ambient temperature also affects the required dose, and buffering increases the rate at which anesthetics take effect. Some chemicals produce initial excitement before anesthesia, and the use of tranquilizers in conjunction with the anesthetic agent may be indicated. Most of the information available on the effects of anesthetics on amphibians focuses on adults. Additional caution must be taken in administering anesthetics to younger age groups, as they have been shown to differ in drug metabolism and skin permeability (Goulet et al., 2010).

Induction and recovery can take over 30 minutes, and animals must be monitored throughout this time to ensure they do not drown (Gentz, 2007). While recovering, amphibians should be placed in fresh water at a temperature suited to the species. Anurans and other non-gilled amphibians must have their nostrils above the surface of the water during induction and recovery (Girling, 2013); this can be accommodated by positioning the anesthetic tank on a slope to produce shallow water at one end. An animal is considered to be recovered when it can swim or walk normally.

When an animal is anesthetized, relevant information, such as the type of anesthetic, method of administration, and any complications or welfare concerns, must be documented in the animal records. Wright and Whitaker (2001) provide useful information on amphibian anesthesia, including anesthetic protocols.

10.10.1.1 Anesthetics

Tricaine methanesulfonate (TMS, also known as MS222) is a common anesthetic for amphibians; however, it must be buffered and only used on healthy animals, with the dose calibrated for the species, life stage, and size of the animal. While different grades of TMS are available, the veterinary formulation should be used. Immersion in TMS is the preferred route, but injection may be suitable for some species and situations (Koustubhan et al., 2013).

The literature on the use of local anesthetics in amphibians is very limited, and few experimental studies evaluate their efficacy (Chatigny et al., 2017). Lidocaine can provide the same anesthetic effect as TMS; however, these drugs have similar properties, and administering lidocaine to animals that have been anesthetized with TMS does not provide additional benefit to the animals. Care must be taken to ensure the animal is not overdosed.

In a comparison of the anesthetic effects of benzocaine and TMS in both terrestrial and aquatic adult forms of *Ambystoma tigrinum nebulosum*, benzocaine was found to induce anesthesia more quickly but had a longer and more variable recovery time (Crook and Whiteman, 2006).

While some studies support the effectiveness of eugenol (Goulet et al., 2010), pharmaceutical-grade eugenol is not available, and the substance has not been approved for use in Canada.

10.10.1.1.1 Immersion

The skin of amphibians is highly permeable, and therefore soaking in a solution of a soluble anesthetic agent is an effective method to induce anesthesia. The absorption of the drug is influenced by the surface area of the animal's body, which is related to its weight (Goulet et al., 2010).

Because TMS is acidic when dissolved in water, the solution imposes stress on the animal, and the majority of the tricaine converts into a form that cannot be absorbed (Wright and Whitaker, 2001a). TMS should therefore be buffered with sodium bicarbonate (Gentz, 2007) or another suitable buffering agent (e.g., HEPES, Tris-Base, or sodium-phosphate). Wright and Whitaker (2001) and Girling (2013) provide recommended dosages and buffering procedures, which depend on the concentration of TMS. The final solution should have a pH of 7.0-7.4 (Gentz, 2007; Green, 2010; Reed, 2005).

When using an anesthetic solution, as soon as the animal becomes unresponsive to the eyelid-touch and toe-pinch tests, it must be removed from the solution (Gentz, 2007). When anesthetized animals are held out of the water, they should be kept moist to allow respiration through the skin. For lengthy procedures, the animal should be transferred to a solution that contains half the concentration of the solution used to induce anesthesia, or the animal should be sprayed with the anesthetic solution to ensure it remains anesthetized.

Loss of righting reflex indicates light anesthesia, whereas loss of withdrawal reflex to nociceptive stimulus indicates deep anesthesia (Gentz, 2007). Heart rate and breath rate must be monitored to determine the level of anesthesia. For some species, monitoring can be done through observation (e.g., in Anurans, the floor of the mouth moves with each breath and the heart beat may be seen through the skin when placed on dorsum); however, for others monitoring equipment (e.g., a Doppler oximeter or ECG) is required. If the anesthetic level becomes too deep, as determined by heart rate monitoring, the animal must be rinsed with clean well-oxygenated water until recovery (Gentz, 2007).

Usually, amphibians recover from TMS within 30-90 minutes after being rinsed in freshwater. Anesthetic solutions should be discarded as chemical waste.

10.10.1.1.2 Injection

Injectable anesthetics such as propofol have been tried on frog species (von Esse and Wright, 1999); however, Green (2010) does not recommend injectable propofol as a safe option for *Xenopus*.

10.10.1.1.3 Hypothermia

Hypothermic anesthesia is inappropriate for amphibians as it does not appear to induce rapid unconsciousness in these animals, and the level of anesthesia is difficult to monitor (Martin, 1995). However, in some situations, low temperature may be appropriate as a supplement to other forms of anesthesia (Lillywhite et al., 2017).

10.10.1.2 *Xenopus*

Buffered TMS is the most common anesthetic for *Xenopus* and can be administered by immersion (300-500 mg/L water) or injection (50-150 mg/kg) (Wolfensohn and Lloyd, 2003, cited in Reed, 2005). Immersion in buffered TMS for 20 minutes has been shown to provide effective anesthesia in *Xenopus laevis* for procedures lasting less than 30 minutes when the concentration is 1 g/L, or 60 minutes when a concentration of 2 g/L is used (Lalonde-Robert et al., 2012).

Induction with TMS can occur in as little as 5 minutes (Reed, 2005) but can take 10-20 minutes (Green, 2010). Anesthesia is assessed by loss of righting reflex, loss of corneal reflex, and loss of withdrawal response to toe pinch (Green, 2010). There is also a reduction in respiratory effort, with slowing of throat movements (Reed, 2005).

While immersion in buffered TMS is the preferred method, immersion in benzocaine can be an alternative for *Xenopus* (Smith et al., 2018). Some other anesthetics that have been used on *Xenopus* are only effective long enough for short procedures (e.g., topical application of liquid isoflurane) or leave the animals sensitive to pain and are only suitable for minor procedures (e.g., ketamine) (Reed, 2005). In addition, the aversive behaviour and risk of fatality associated with other forms of isoflurane suggest further information is needed regarding its topical application (Reed, 2005). While some studies support the effectiveness of eugenol (Goulet et al., 2010), pharmaceutical-grade eugenol is not available, and the substance has not been approved for use in Canada. Guénette et al. (2013) provide a review of the effectiveness of a number of anesthetics that have been used in frogs.

As noted above for all Anurans, *Xenopus* must have their nostrils above the surface of the water during induction and recovery.

10.10.1.3 Axolotls

Buffered TMS is commonly used to anesthetize axolotls. For procedures lasting 15-30 minutes that require anesthesia, immersion of axolotls in 0.2% TMS solution (i.e., 2 g/L) for 20 minutes can be used (Zullian et al., 2016). A weaker TMS solution of 0.1% does not produce anesthesia within the same timeframe (Zullian et al., 2016). For procedures requiring a longer period of anesthesia, such as amputation of a limb in regeneration studies, a higher concentration of 0.3-0.4% may be appropriate (Zullian et al., 2016).

The depth of anesthesia can be determined by the withdrawal reflex test or the righting reflex test (Zullian et al., 2016).

During recovery, axolotls should be continually monitored and kept at a temperature no greater than 23 °C.

10.10.2 Analgesia

Guideline 22

Following the precautionary principle, amphibians should be provided with analgesia for procedures that are likely to be painful, based on the best available scientific evidence.

Analgesia is encouraged where relevant information can be found. The inability of observers to recognize the nociceptive response or detect distress should not be justification for withholding analgesia.

In the absence of relevant information on analgesics, there may be unknown side-effects for the animals and impacts on the research. In the case of axolotls, it is unknown if analgesia will interfere with regeneration studies, and it may not benefit the animals, as they do not display behaviours generally associated with pain following amputation (e.g., they exhibit normal feeding behaviour and do not avoid the use of the amputated limb). A study has shown no behavioural difference in response to an acute noxious stimulus when axolotls were given analgesics (Llaniguez et al., 2020); however, more research in the area of effective analgesic regime is needed.

There is limited information on analgesia in amphibians; however, recommendations on the use of some analgesics in particular species are reviewed by Stevens (2011) and Gentz (2007). Careful evaluation of the dosage is important; for example, a low dosage of flunixin meglumine (25 mg/kg) has been found to provide effective analgesia in *Xenopus*, but higher dosages are associated with fatalities (Smith et al., 2018).

10.11 SURGERY

Amphibians should be fasted before surgery, with the length of time based on the particular animal. Gentz (2007) recommends at least 4 hours for small amphibians, 48 hours for large insectivorous amphibians, and one week for amphibians that consume whole vertebrate prey.

Amphibians must be kept hydrated during surgery. Aquatic and semi-aquatic amphibians should be placed in water for 1 hour before surgery and kept at least partly in water during surgery (Gentz, 2007).

Surgical preparation should involve gently rinsing or flushing the surgical site with sterile saline solution. Amphibian skin should not be rubbed. The skin has natural antibiotic properties, which reduce the need for surgical site disinfection. If required, a 0.75% chlorhexidine solution can be used (Gentz, 2007), but it is generally not preferable. Soaps, detergents, isopropyl alcohol, and iodine must not be used on amphibians.

Adhesive drapes must not be used (Gentz, 2007).

Antibiotics may be necessary for some types of surgery; however, their use should be carefully considered in consultation with the veterinarian. For axolotls, some antibiotics can inhibit matrix metalloproteinases (MMPs), which are essential for proper wound healing.

10.12 MONITORING AND POST-PROCEDURAL CARE

Amphibians that are primarily aquatic must be rinsed thoroughly and placed in water that is free of anesthetic (Gentz, 2007). The water temperature should be the same as that of the animal's housing environment or cooler, depending on the species and situation. For axolotls that have undergone major surgery, a water temperature of 4 °C for up to 40 hours can help with recovery.

Subcutaneous or intracoelomic fluids can be given postoperatively when necessary (Gentz, 2007). Depending on the type of surgery and species, it may also be necessary to assist the animals with eating (Gentz, 2007). Suggestions for formulations of postoperative fluids and nutritional options for amphibians, particularly *Xenopus*, are provided by Gentz (2007).

Antibiotic therapy may be necessary but should only be provided when advised by the veterinarian.

11 EUTHANASIA

The General Guiding Principles outlined in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) apply to euthanasia of all animals in science. This section provides additional information that is specific to euthanasia of amphibians.

Guideline 23

Euthanasia of amphibians must be carried out only by competent personnel using the least invasive method that is suited to the particular species, life stage of the animal, and the study objectives.

For all methods of euthanasia:

- personnel involved in the procedure must be trained and have their competency assessed with regard to their performance of the procedure on the particular species involved and their ability to confirm the death;
- equipment must be appropriately maintained and cleaned before re-use;
- any animals undergoing euthanasia must not be left unattended prior to confirmation of death;
- animals must not be mixed with unfamiliar animals prior to euthanasia; and
- stress caused by handling should be minimized.

For commonly used amphibians, such as *Xenopus* and axolotls, the AVMA recommends applying a physical method of euthanasia on fully anesthetized animals (AVMA, 2020). However, other methods are considered acceptable, as noted in the sections below. For all methods of euthanasia, a second method should be applied to ensure death, as it can be difficult to confirm death in these animals (AVMA, 2020).

11.1 IMMERSION OR TOPICAL AGENTS

The *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) notes that immersion of amphibians in buffered TMS or benzocaine is acceptable. AVMA (2020) recommends prolonged immersion (one hour) in a buffered TMS solution with a concentration of 5-10 g/L. Benzocaine hydrochloride can be applied through immersion at a concentration ≥ 250 mg/L or as a topical gel (7.5% or 20%) on the ventral surface of an amphibian, with the dose based on the species and size of the animal (AVMA, 2020).

For *Xenopus*, immersion in an overdose of TMS is preferred as it appears to be one of the least stressful methods for the animals (Reed, 2005). Immersion for at least one hour in a buffered solution with a concentration of 5 g/L TMS is required for euthanasia of *Xenopus laevis* (Torreilles et al., 2009). The application of 20% benzocaine gel to the ventral skin of *X. laevis* is an alternative method that results in death after 3-5 hours (Torreilles et al., 2009). For both methods, death should be confirmed through the application of a secondary physical method.

11.2 INJECTION

Since amphibians readily absorb chemicals through the skin, euthanasia can often be accomplished through immersion or topical agents, followed by a physical method. However, for situations where injection is necessary, the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) lists injection of buffered TMS, injection of benzocaine, and SC injection of barbiturates into the lymph sac as acceptable methods for frogs and toads.

Intravenous injections (via the ventral caudal tail vein or the ventral abdominal vein) are acceptable but can be difficult due to the small size of the animals and the difficulty in accessing blood vessels. Intraperitoneal injections are also acceptable. Other acceptable routes include subcutaneous lymph spaces and lymph sacs (AVMA, 2020). Appropriate doses and time to loss of consciousness and death depend on the agent, route, and the species and size of the animal.

For *Xenopus*, an overdose of pentobarbital by injection via intracardiac (under anesthetic), intracoelomic, or subcutaneous into the lymph sac is recommended (Gentz, 2007). The dosage required depends on the species; for example, Torreilles et al. (2009) recommend intracoelomic injection of 1100 mg/kg sodium pentobarbital with sodium phenytoin for *Xenopus laevis*.

11.3 INHALANT ANESTHETICS

Inhalant anesthetics are not acceptable for *Xenopus* (Green, 2010), and they are generally not effective for other amphibian species. If inhalant anesthetics are being considered, a pilot study should be undertaken to understand their effectiveness and welfare impacts on the species of interest.

An overdose of inhalant anesthetic is not appropriate for species that can hold their breath and are therefore resistant to the action of the anesthetic. If used on other species, the procedure must be followed by another method to ensure death (CCAC, 2010). Inhalant anesthetics may cause initial excitement, and some are irritants or perceived as noxious to the animals (AVMA, 2020). Due to the long exposure time required to achieve death, a physical method of euthanasia must be applied after the loss of consciousness.

11.4 PHYSICAL METHODS

The *AVMA Guidelines for the Euthanasia of Animals* (AVMA, 2020) recommends that amphibians be euthanized by a physical method while fully anesthetized.

For *Xenopus*, decapitation of an anesthetized animal is an acceptable physical method (Green, 2010). Pithing is also acceptable if preceded by TMS (immersion or IC injection) and followed by decapitation as a secondary method (Green, 2010). Decapitation or pithing without anesthesia is not acceptable (Reed, 2005); for decapitation, brain function can continue for an extended period.

Similar methods are acceptable for anesthetized axolotls (i.e., TMS followed by pithing or decapitation).

11.5 OTHER METHODS

When the methods described above cannot be used, the veterinarian should be consulted to determine the most appropriate alternative method.

Concussion of the brain by striking the cranium, followed by pithing or decapitation before the return of consciousness, is conditionally acceptable when other methods cannot be used (AVMA, 2020).

Hypothermia and rapid freezing should not be used to euthanize amphibians unless specific scientific justification is provided and the procedure is approved for the study by the animal care committee. However, rapid freezing in liquid nitrogen followed by a second method of euthanasia to ensure death may be acceptable for animals weighing <4 g, for species that are not adapted to freeze tolerance strategies (AVMA, 2020).

Other methods of euthanasia may be appropriate for euthanasia of amphibians in field settings; however, their use in the laboratory requires scientific justification and the approval of the animal care committee.

11.6 EUTHANASIA OF LARVAE

Once larvae have developed beyond exclusive reliance on their own yolk nutrients (i.e., have begun external feeding), they are covered by these guidelines. In general, amphibian larvae at this stage should be euthanized using the same methods as adults (AVMA, 2020). They can be immersed in buffered TMS, followed by pithing or concussion to ensure death where feasible, based on the size of the larvae. Freezing larvae in liquid nitrogen is also an option. Larger larvae should be anesthetized first, but it is not necessary for small larvae as the time to death in liquid nitrogen is very short. If unsure, the veterinarian should be consulted.

12 END OF STUDY

12.1 TRANSFER OF AMPHIBIANS BETWEEN FACILITIES OR PROTOCOLS

For amphibians that are to be transferred to another institution at the end of a study, see Section 4, "Procurement", particularly with regard to regulations, documentation, and transportation. As mentioned, this applies to amphibians that have not been subject to major invasive procedures and are fit to travel.

If amphibians are transferred to an institution that is not CCAC-certified, it is the responsibility of the institution sending the amphibians to ensure the animals will receive appropriate care.

12.2 RE-HOMING

Where permitted by regulatory authorities, institutions may release healthy research amphibians (although not those that have been genetically modified in the laboratory) that are commonly accepted pet or companion species to individuals who have the knowledge and ability to provide proper care to the animals. Amphibians that have been genetically modified may not be moved from research facilities to private premises. As with any other animals, if amphibians are to be released to the care of an individual as companion animals, the institution should develop an appropriate policy describing the conditions that need to be fulfilled before the release of the animals. Institutions should ensure those who will be adopting the amphibians are aware of the care required. Records should be kept, and new owners should be informed of their responsibility to contact the institution if an animal exhibits a delayed reaction to research procedures and to accept the direction of the institution in regard to the situation.

12.3 RELEASE TO THE WILD

The release of captive wildlife, including amphibians, is discussed in the *CCAC guidelines on: the care and use of wildlife* (CCAC, 2003b). Release of any animal must adhere to federal, provincial/territorial, and local laws and regulations. In addition, there must be an evaluation of the benefits and risks to the animal, to other animals at the release site, and to the ecological conditions of the release site. No genetically modified amphibians may be released from research facilities.

12.4 DISPOSAL OF DEAD AMPHIBIANS

Amphibian carcasses must be disposed of according to the relevant federal, provincial/territorial, and municipal regulations for the disposal of biological materials.

13 HUMAN SAFETY

Institutions have occupational health and safety programs that are specifically tasked with addressing this topic through risk assessments. The responsibility of the animal care committee extends to ensuring that there is an institutional occupational health and safety program in place so that any risks to human health and safety are properly assessed and communicated to all personnel working with these animals.

Those working with animals must follow institutional policies and SOPs outlining appropriate measures of prevention and protection. They should seek professional knowledge on animal allergens, zoonotic diseases, and other risks or hazards that may be associated with a particular study (e.g., exposure to radiation, anesthetic gas, chemical hazards, and human cell lines).

People working with amphibians should take precautions against bites and scratches, as appropriate. In addition, caution should be taken when using needles or sharp instruments on amphibians, as their small size may increase the risk of personnel poking or cutting themselves.

There are several pathogens that can be transmitted between amphibians and humans (zoonoses), such as salmonella, *Escherichia coli*, and *Edwardsiella tarda*. Handling protocols should include handwashing between handling different animals and immediately after handling the animals.

Skin secretions of *Xenopus* and many other amphibians are toxic, and people working with these animals must ensure secretions do not come in contact with their eyes (Tinsley, 2010). In addition, powderless nitrile gloves can protect the handler's skin against these secretions without leaving residues or irritants that could harm the animals. Goggles may also be necessary when working with species that are able to squirt toxins (e.g., the giant toad, *Bufo marinus*) (Girling, 2013).

Many amphibians have developed various adaptive toxic strategies for protection against predation. While some species are inherently poisonous, others like *Dendrobates* gain that ability from prey items. The latter gradually lose their toxic properties when raised in captivity if they do not have access to the toxins in the food items. However, any wild-sourced animals should be treated with caution in this regard, and a good knowledge of the species is important.

While allergic reactions to laboratory amphibians are not common, it is possible that sensitivity to amphibian proteins could develop, and precautions should be taken, such as wearing gloves and handwashing (see the UC Davis Information Sheet on the Care and Use of Amphibians for more details).

Facilities housing amphibians can be subject to wet floors, which present a physical hazard for personnel. SOPs must be in place to minimize any associated risks.

Personnel who will be moving tanks should be trained in ergonomically correct methods. Tanks with soil or water are heavy and require proper preparation for moving (e.g., an aquarium more than one-quarter full is significantly more likely to shatter when moved than one with less water).

Precautions must also be taken when working with substances such as TMS, which is irritating to the eyes, skin, and respiratory tract. This includes ensuring SOPs for handling such substances are followed, wearing appropriate personal protective equipment, and working in a fume hood when possible.

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APPENDIX 1
COMMONLY HELD SPECIES AND ADDITIONAL RESOURCES

COMMONLY HELD SPECIES

Frogs

- *Lithobates catesbeianus (Rana catesbeiana)*, bullfrog
- *Lithobates (Rana) pipiens*, leopard frog
- *Lithobates (Rana) sylvatica*, wood frog
- *Xenopus laevis* and *Xenopus (Silurana) tropicalis*, African clawed frog

Toads

- *Anaxyrus americanus*, American toad
- *Rhinella marina*, cane toad

Salamanders

- *Ambystoma mexicanum*, axolotl
- *Ambystoma tigrinum*, tiger salamander
- *Notophthalmus viridescens*
- *Plethodon* spp., woodland salamanders

Caecillians

ADDITIONAL RESOURCES

Biology and Natural History

Amphibians

O'Rourke D.P. and Rosenbaum M.D. (2015) Biology and diseases of amphibians. In: *Laboratory Animal Medicine,* 3rd ed. (Fox J., Anderson L., Otto G., Pritchett-Corning K. and Whary M., eds.). Chapter 18**.** Cambridge MA: Academic Press.

Wells K.D. (2007) *The Ecology and Behavior of Amphibians*. Chicago IL: University of Chicago Press.

Xenopus

Green S.L. (2009) *The Laboratory* Xenopus *sp.*, 1st ed. Boca Raton FL: CRC Press.

Axolotls

Armstrong J.B. and Malacinski G.M. (1989) *Developmental Biology of the Axolotl*. New York NY: Oxford University Press.

Housing, Husbandry, and Breeding

Amphibians

Wright K.M. and Whitaker B.R. (2001) *Amphibian Medicine and Captive Husbandry*. Malabar FL: Krieger Publishing Company.

Xenopus

Green S.L. (2009) *The Laboratory* Xenopus *sp.*, 1st ed. Boca Raton FL: CRC Press.

Sive H.L., Grainger R.M. and Harland R.M. (2000) *Early Development of* Xenopus laevis: *A Laboratory Manual*. 338 pp. Woodbury NY: Cold Spring Harbor Laboratory Press.

Axolotls

Farkas J.E. and Monaghan J.R. (2015) Housing and maintenance of *Ambystoma mexicanum*, the Mexican axolotl. *Methods in Molecular Biology* 1290:27-46.

Khattak S., Murawala P., Andreas H., Kappert V., Schuez M., Sandoval-Guzman T., Crawford K. and Tanaka E.M. (2014) Optimized axolotl (*Ambystoma mexicanum*) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nature Protocols* 9(3):529-540.

Enrichment and Welfare

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Kuppert S. (2013) Providing enrichment in captive amphibians and reptiles: Is it important to know their communication? *Smithsonian Herpetological Information Service* 142:1-42.

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Morgan K.N. and Tromborg C.T. (2007) Sources of stress in captivity. *Applied Animal Behaviour Science* 102(3-4):262-302.

Xenopus

Holmes A.M., Emmans C.J., Coleman R., Smith T.E. and Hosie C.A. (2018) Effects of transportation, transport medium and re-housing on *Xenopus laevis* (Daudin). *General and Comparative Endocrinology* 266:21-28.

Health and Disease Control

Amphibians

O'Rourke D.P. and Rosenbaum M.D. (2015) Biology and diseases of amphibians. In: *Laboratory Animal Medicine*, 3rd ed. (Fox J., Anderson L., Otto G., Pritchett-Corning K. and Whary M., eds.). Chapter 18. Cambridge MA: Academic Press.

Xenopus

Green S.L. (2010) *The Laboratory* Xenopus *sp.*, 1st ed. Boca Raton FL: CRC Press.

Experimental Procedures

Amphibians

Wright K.M. and Whitaker B.R. (2001) *Amphibian Medicine and Captive Husbandry*. Malabar FL: Krieger Publishing Company.

Xenopus

Green S.L. (2010) *The Laboratory* Xenopus *sp.*, 1st ed. Boca Raton FL: CRC Press.

Administration and Removal of Substances

Axolotls

Debuque R.J. and Godwin J.W. (2015) Methods for axolotl blood collection, intravenous injection, and efficient leukocyte isolation from peripheral blood and the regenerating limb. *Methods in Molecular Biology* 1290:205-226.

Genetic Modification

Xenopus

Sive H.L., Grainger R.M. and Harland R.M. (2000) *Early Development of* Xenopus laevis*: A Laboratory Manual*. 338 pp. Woodbury NY: Cold Spring Harbor Laboratory Press.

Salamanders

Kumar A. and Simon A. (eds.) (2015) *Salamanders in Regeneration Research: Methods and Protocols*. 357 pp. New York NY: Humana Press.

Axolotls

Armstrong J.B. and Malacinski G.M. (eds.) (1989) *Developmental Biology of the Axolotl*. Oxford UK: Oxford University Press.

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Khattak S., Murawala P., Andreas H., Kappert V., Schuez M., Sandoval-Guzman T., Crawford K. and Tanaka E.M. (2014) Optimized axolotl (*Ambystoma mexicanum*) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nature Protocol* 9(3):529-540.

Khattak S. and Tanaka E.M. (2015) Transgenesis in axolotl (*Ambystoma mexicanum*). *Methods in Molecular Biology* 1290:269-277.

Anesthesia, Analgesia and Surgery

Axolotls

Zullian C., Dodelet-Devillers A., Roy S. and Vachon P. (2016) Evaluation of the anesthetic effects of MS222 in the adult Mexican axolotl (*Ambystoma mexicanum*). *Veterinary Medicine* (Auckland N.Z.) 7:1-7.

Specific Areas of Research – Regeneration

Salamanders

Kumar A. and Simon A. (eds.) (2015) *Salamanders in Regeneration Research: Methods and Protocols*. 357 pp. New York NY: Humana Press.

APPENDIX 2
METHODS OF IDENTIFICATION

METHOD	ADVANTAGES	DISADVANTAGES	REFERENCES
PREFERRED METHOD			
Natural marking	• Non-invasive • Inexpensive • Useful for small numbers of animals • Facilitated by easily accessed digital photography (e.g., smart phones)	• Time consuming for large numbers of animals • Markings fade when an animal dies • Patterns in very young or albino animals are not easy to see • Skin colour can alter due to stress or environmental fluctuations (lighting, humidity, temperature) and make the pattern recognition harder • Patterns can change over time, depending on background colour, season, age, etc. • There is a cost if using image-recognition software	Reed, 2005 Green, 2010 Schoen et al., 2015
Visible implant elastomer (VIE)	• Successfully used in frogs and salamanders • Many colours are available and site of injection can vary for more possibilities • Only a small volume of material is necessary for a visible tag • Can be used in smaller animals than many other marking techniques • VI Alpha Tags are similar but have alphanumeric codes	• Marks may migrate and become lost • Low visibility of marks in some species due to skin pigmentation • Needs to be kept cold until immediately prior to injection • Initially relative expensive	Sapsford et al., 2015 Antwis et al., 2014

METHOD	ADVANTAGES	DISADVANTAGES	REFERENCES
MIGHT BE SUITABLE IN CERTAIN CIRCUMSTANCES			
Microchip transponders	• Large numbers of animals can be individually identified • Effective, practical, and reliable for the frog's life, normally without adverse effect • Chips can be sterilized and re-used • Immediate skin tissue damage should be minimal	• Cannot distinguish between animals with the naked eye • Animals normally have to be removed from water to be scanned • Difficult to track identity of a single animal in large groups • Potential (although rare) for microchips to migrate or be expelled • Some concern of ulcers or inflammatory responses occurring around injection site • Expensive • For some species (e.g., axolotls), it is only feasible when large numbers are kept for breeding purposes • Not suitable for small species	Reed, 2005 Green, 2010
Plastic beads sewn onto the leg or back; studs or tags attached to the web of feet	• No reported perceived health or welfare complications	• Requires anesthesia • Skin and feet web in particular are very delicate, containing many blood vessels which may be damaged • Could disrupt mucous membrane of the skin and possibly increase the risk of infection • Risk of mark falling off, being ingested, or causing constriction • Beads/tags could get caught (therefore not recommended for field marking)	Reed, 2005 Green, 2010
Injected Decimal Coded Wire Tag		• Require magnification to read	
Radio-active markers	• Used for multiple amphibians		Silvy, 2012

METHOD	ADVANTAGES	DISADVANTAGES	REFERENCES
SHOULD NOT BE USED			
Freeze branding	• Only potentially acceptable for field studies where alternatives are fewer	• Significant adverse effects • Frogs shed skin and brand may be illegible within a few months to a year	Reed, 2005 Green, 2010
Tattooing		• Requires anesthesia • Vibrating needles and disruption of the protective slime layer and dermis may predispose animals to disease • Often need to re-tattoo due to skin shedding • Bruising, redness and swelling have been observation of for days after tattooing	Reed, 2005 Green, 2010
Applying acetic acid to the skin		• Painful for the animal	Reed, 2005
Leg ringing		• If bands are tight enough to remain attached, they are probably too tight and could restrict blood circulation, causing swelling and necrosis	Reed, 2005
Removal of toes or fingers		• Causes tissue damage and pain; may cause distress • Could be detrimental to health; appears to decrease survival in the wild • Digits can re-grow, particularly in axolotls	Reed, 2005
Skin grafts		• Requires anesthesia and surgical expertise • Limited to small number of individuals • No information on health or welfare issues or longevity of the method • Very complicated for identification purposes	Reed, 2005

GLOSSARY

Acclimation – a persisting physiological, biochemical, or morphological change within an individual animal during its life as a result of a prolonged exposure to an environmental condition such as a high or low temperature; generally, the changes are reversible.

Aestivation – a state of dormancy in response to high temperature and dry conditions.

Airstone – a device added to a system to gradually aerate the water.

Amplexus – a mating position for some externally fertilizing species in which the male grasps the female from behind.

Analgesia – decrease in response to noxious stimuli.

Anesthesia – a state caused by an external agent leading to loss of sensation and motor function.

Aseptic – absence of living germs, free from septic and poisonous putrefactive products.

Chloramine – a chemical compound that contains chlorine and ammonia, often found in municipal water supplies.

Competency – competency refers to the ability to effectively perform a particular task in relation to the care, maintenance, or use of the animals, while ensuring their welfare is protected as far as possible within the constraints of any approved studies that the animals are involved in. Focusing on competency rather than training acknowledges that there may be a variety of ways of acquiring the necessary knowledge and skills, and places emphasis on learning outcomes. See *CCAC guidelines on: training of personnel working with animals in science* (CCAC, 2015) for more details.

Conspecifics – animals belonging to the same species.

Crypsis – the ability of an animal to conceal itself from detection by other animals.

Distress – a state where the animal must devote substantial effort or resources to the adaptive response to challenges emanating from the environmental situation; it is associated with invasive or restrictive procedures conducted on an animal or other conditions that significantly compromise the welfare of an animal, which may or may not be associated with pain.

Ectothermic – an animal that assumes the temperature of its surroundings.

Endpoint – predetermined criteria for intervening in a procedure to terminate, minimize, or reduce an animal's pain and/or distress, which takes into account the welfare of the animal (welfare endpoint) and the goal of the experiment (scientific endpoint).

Environmental enrichment – enhancements to an animal's environment that go beyond meeting its basic species-specific needs and further improve overall quality of life.

Fecundity – the capability to produce offspring.

Fomites – non-living objects that can carry disease organisms (e.g., mops).

Genetically modified – a deliberate modification of the genome (the material responsible for inherited characteristics).

Genotyping – a process used to determine differences in the genetic makeup (genotype) of an individual animal by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.

Hypothermia – lower than normal body temperature.

Intraperitoneally (intracoelomically) – within the peritoneum or body cavity.

Lateral line – a sensory system running along the side of some amphibians that detects movement, vibration, and pressure.

Metamorphosis – the process of undergoing a distinct physical change in developing from larva to adult.

Microbiome – a community of microorganisms within a defined environment (e.g., the microorganisms inhabiting the skin of an animal).

Morbidity – visible manifestation of a diseased state.

Mortality – loss of life; death.

Nociception – the process of communication through the nervous system when noxious stimuli have been detected by particular receptors (nociceptors).

Noxious stimuli – those stimuli that are damaging or potentially damaging to normal tissue.

Oocyte – an immature egg cell.

Osmoregulatory – a system regulating the body fluids of an organism to maintain an optimum concentration of electrolytes.

Pain (in amphibians) – amphibian pain is a response to a noxious stimulus that results in a change in behaviour or physiology and the same noxious stimulus would be painful to humans (a working definition).

Personal protective equipment (PPE) – garments or equipment designed to protect personnel from injury, infection, or allergic reaction when working with animals; potential hazards include physical injury (bites, scratches, etc.), biohazards, and airborne particulate matter.

Phenotype – refers to the observable physical properties of an organism; these include the organism's appearance, development, and behaviour.

Quarantine – confinement of animals that may carry an infectious disease for a specified period to allow for evaluation.

Sentinel animals – specific pathogen-free (SPF) animals known to be susceptible to an infectious agent that are placed in the area suspected of being contaminated, for example in a new shipment of animals under quarantine; the sentinel animals are then tested for infection or development of antibodies to the infectious agent.

Standard operating procedure – written documents that describe in step-by-step detail how a procedure should be carried out.

Stress – a state caused by factors external to an animal that displace homeostasis; stress can be beneficial (e.g., in triggering a flight response if the animal is threatened, thus helping it to cope with changes in its environment); however, prolonged stress can cause changes to an animal's endocrine system, leaving it less able to cope with its environment.

Three Rs – Replacement, Reduction, and Refinement in animal-based science, as first explained by Russell and Burch in *Principles of Humane Experimental Technique* (1959).

Torpor – an inactive state, often associated with low body temperature and low metabolic rate in response to reduced food availability.

Welfare – the physical health and mental well-being of the animal.

Zoonotic – relating to the transmission of a disease from a non-human species to humans.