Additional Information on Aquatic Species

Additional Information on Observations:

Fish: Healthy fish should swim in a relaxed motion orientated parallel to the surface of the water. Normal behavior includes effortless breathing (movement of the operculum) and an interest in activity such as swimming and eating. Signs of illness can include poor body condition (low ratio of body weight in grams to the cube of the body length in cm), rapid breathing, obvious lesions (masses, ulcers, wounds), and changes in color, shape, or locomotion. Sharp turns, spinning, listing (leaning to one side) or bobbing (fish oriented perpendicular to surface) are also signs of illness. Some of the clinical signs may be expected due to random genetic mutations perpetuated by the research or more specifically a targeted transgene in transgenic animals.

Frogs: Amphibians are ectotherms and cannot raise their body temperature by producing metabolic heat. They must rely on external heat sources. All species have a preferred body temperature range (PBT) at which they optimally function. Tadpoles prefer slightly warmer temperatures. *Xenopus laevis* are pale to dark grey or green dorsally with an off white underside. Their average size is 8-15 cm. Their skin is smooth and very slippery due to a protective mucous covering. Healthy frogs may spend most of their time lying motionless below the surface of the water. Signs of sickness include moving slowly, spending excessive time the surface of the water, inability to dive, or staying on the bottom of the cage. Additional clinical signs may include bloating, shedding large amounts of skin, tufts of cottony white material growing on the skin, red spots or streaks, or swollen and/or reddened body appendages. Any animals exhibiting anorexia should be noted and carefully monitored. Animals with clinical signs of disease may need to be separated or quarantined. Body condition may be evaluated by looking for prominence of the skeleton and palpation of musculature and abdominal contents. Weight is highly variable and depends on the state of hydration; amphibians can lose up to 50% body weight in fluids before death.

Additional Information on Diagnostics and Treatments:

Fish: Skin scrapings and gill biopsies are useful to detect fungal, bacterial and parasitic infections. In general, unless the fish are held at or near their optimum lethal temperature, the water temperature should be elevated 5°C and treated with iodized salt at a concentration of 3 g/l. Salt treatment is effective against fungi, protozoa, and other eukaryotes, as well as bacterial infections. Most freshwater fish can tolerate concentrations as high as 10% and cichlids can tolerate concentrations as high as 60%, which is roughly twice the saline concentration as seawater. Salt treatment also has the advantage in that the salt is completely purged from the fish's body, once they are returned to freshwater. Alternatively, fish can be treated with antibiotic in consultation with the ULAR veterinary staff. If necessary, fish may be euthanized with an overdose of buffered MS222. The tank where sick fish have been held should be cleaned with dishwashing liquid, and soaked in a Clorox solution for 24 hours then rinsed well. Any nets used to capture diseased fish should be rinsed or soaked in a commercial net soak product, potassium permanganate (< 2ppm), an aquarium salt solution or 1:20 bleach immediately after being used and rinsed thoroughly. If sick fish are housed in an area that houses healthy fish, special care should be taken not to contaminate water from one tank with that from another.

Frogs: Skin scrapings and impression smears are useful to detect fungal, bacterial and parasitic infections. Focal and nodular lesions should be biopsied, Gram's and acid-fast stained and

cultured. Hematology has limited value because of the lack of normal data, plus wide variations due to sex, season, and hydration levels. Culture and sensitivity of lymph can be utilized to diagnose bacterial systemic infections. A fecal exam can detect protozoan and metazoan parasites, but significance depends upon the type and extent of infestation with presence of clinical signs. Radiology can be useful to find skeletal deformities, gastrointestinal impactions, foreign bodies or pneumonia. Fiberoptics can be used to view the stomach, biopsy internal organs or for sex identification. Transillumination of small, thin skinned amphibians by means of a cool fiberoptic source can be used to view the lungs, ova and other coelomic contents. Frogs can be treated with antibiotic in consultation with the ULAR veterinary staff. If necessary, frogs may be euthanized with an overdose of buffered MS222.

Additional Information on Receiving New Animals and Quarantine:

The shipment water should be kept with the new arrivals and gradually be replaced by slow dilution over several days with facility water to avoid "shock" to new animals. This may be as simple as separate cages for new animals or it may mean separate animal rooms. The purpose of quarantine is to allow animals to stabilize following shipment, to avoid exposure to incoming disease from new stock, to condition new animals to existing environmental conditions prior to commencement of an experiment, and to seek replacement animals for sick or damaged animals from the vendor if necessary.

Personnel providing care should tend first to established healthy collections and breeding colonies, and newly arrived animals should be handled last. Separate holding tanks and handling equipment (nets, scoops, etc.) should be kept for quarantine animals. Caretakers should adhere to frequent glove changes and/or hand washing procedures between tanks of different shipments and between individual animals whenever feasible.

Frogs: Frogs particularly should be examined daily for hemorrhages on the legs. Preventative treatments for newly shipped animals may include placing frogs in a 0.6% calcium hypochlorite solution (or a 0.06% sodium chloride solution) to reduce the growth of *Aeromonas hydrophilia* and occurrence of "Red Leg" caused by the stress of shipment.

Additional Information on Anesthesia:

Fish: Fast fish for 12–24 hours prior to anesthesia to reduce fecal contamination and risk of regurgitation. Maintain adequate oxygenation during anesthesia by supplying oxygen (air) via an air supply stone or similar device and oxygenate all water chambers during anesthesia and recovery. Assess for surgical plane of anesthesia by monitoring loss of equilibrium and muscle tone, decreased respiratory rate, no response to stimuli (firmly squeeze at the base of the tail to determine response to stimuli). Evaluate respiratory rate and gill color (gill color should be dark pink to light red), observe movement of the operculum as it opens and closes to assess rate, if respirations become extremely slow or stop, place the fish in anesthetic-free recovery water until respirations resume.

Anesthesia for Fish

Anesthetic agent	Concentration	Induction time (min)	Route	Maintenance	Recovery time (min)
MS 222®[1,2] (Finquel®, tricaine methanesulfonate)	25-100 mg/l in water or dilution of 1:12000 25-35 mg/l water	1-3	Immersion	excellent if re-circulating water, can provide excellent anesthesia for up to 4 hours (1:12000 dilution) Sedation	3-15
Benzocaine	50 mg/l in water Small margin of safety between effective and lethal doses.	1-3	Immersion	excellent	3-15
Metomidate	7.5-15ppm	2-5	IP	good	6-12
CO_2	200ppm	1-2	Immersion	good	5-10
Halothane	Dissolved 0.5-2ml/l water, or may be bubbled through anesthetic chamber to effect	1-3	Immersion	good	3-15
Isoflourane	bubbled through anesthetic chamber to effect	1-3	Immersion	good	3-15
Ketamine	14-18 mg/kg		IP	30 -60 minutes	
Pentobarbital	30 mg/kg or 72 mg/kg		IP	Sedation prolonged (24 hours) anesthesia	

[1]MS-222 is the only FDA approved anesthetic for fish (21 day withdrawal).
[2] MS-222 doses can vary widely between different species of fish.

- Unbuffered MS-222 is acidic and poorly absorbed, resulting in a prolonged induction time and dermal irritation.

Stages of Anesthesia in Fish:

Stage 1: Deep sedation	**Stage 2: Deep narcosis**	**Stage 3: Surgical anesthesia**
Cessation of voluntary swimming; decreased response to stimuli.	Decreased muscle tone; equilibrium loss; appropriate level for fin and gill biopsies.	Slow respiration and heart rate; total loss of activity to stimuli.

Frogs: Fast for a minimum of 4 hours prior to anesthesia to decrease incidence of regurgitation. Fast larger insectivorous frog species for 48 hours, and frogs on a diet of whole vertebrate prey for 1 week. Induce anesthesia in a container that will avoid injury from the animal jumping or falling out. Anesthetic induction may produce an excitement phase. Pulmonary respiration will cease during anesthesia; therefore, respiratory rate cannot be used to monitor anesthetic depth. Cutaneous respiration is sufficient to prevent clinical hypoxia during anesthesia. Monitor heart rate during anesthesia by direct observation (ventral midline, caudal to the shoulders), ECG, ultrasonagraphy or doppler flow detector. It should be noted that normal values for heart rates have not been published.

Allow animal to reach appropriate level of anesthesia for planned procedures. Remove the animal from the anesthetic bath and rinse with fresh water. The animal will remain anesthetized for 10–80 minutes, depending on the method and drug concentration used. Determine full recovery from anesthesia by monitoring when the righting reflex returns and animal is able to move normally. Recovery may take 30–90 minutes after the animal is rinsed with fresh water. Do not raise the amphibian's body temperature above that of normal room temperature in an attempt to speed recovery. Increased body temperature will increase metabolism and oxygen requirements and cutaneous respiration may not be sufficient to maintain adequate oxygenation in this situation. Do not apply alcohol or other preparations that contain alcohol directly to the skin of an amphibian, as absorption of these products through the skin may dissolve normal secretions that protect the animal from dehydration and infections.

Anesthesia Achieved by Immersion in an Anesthetic Solution

Anesthetic Agent	Dose	Comments
MS-222 (tricaine methanesulfonate)	250-500 mg/L of buffered aqueous solution	Tadpoles
	1-2 g/L of buffered aqueous solution	Frogs and salamanders
	2-3 g/L of buffered aqueous solution	Toads
Benzocaine (powder or hydrochloride)	2 g/L of buffered aqueous solution	True toads, spadefoots, and large salamanders (see below)

Stages of Anesthesia in Amphibians:

Stage 1: Induction	Stage 2: Light anesthesia	Stage 3: Surgical anesthesia
Decreased gular movement and diminished withdrawal reflex.	Loss of righting reflex and absence of abdominal respirations.	No withdrawal reflex (toe pinch) and gular movements cease.

Additional Information Survival Surgery:

The glass bead dry heat sterilizer may be used after the tips of the instruments are wiped with sterile saline or water to remove blood or tissue residue. The instruments must be allowed to cool before use to avoid burning tissues.

Skin is easily damaged and the constant mucus production makes skin disinfection difficult.

Preparation for surgery should be limited to gentle sponging away of mucus in the immediate surgical site with sterile saline. Animals may be draped for surgery with a sterile soft, absorbent, moistened drape or a sterile plastic drape. In addition to protecting the surgical site, the drape also serves as a clean location on which surgical instruments may be placed during the procedure. Aseptic procedure should be observed when performing survival surgery.

Synthetic monofilament absorbable suture materials are recommended for use in fish and frogs as multifilament sutures allow for more wicking of contaminants into the incision from surrounding water. Depending on the surgery, a one or two layer closure may be appropriate. If non-absorbable suture is used for skin closure, sutures should be removed 10-14 days postoperatively.

Frogs: Surgical Harvesting of Oocytes. A 1 to 1.5 cm incision is made in the abdomen, lateral to the midline. A piece of ovary containing several dozen oocytes is then resected. A single absorbable suture may be used to control hemorrhage if necessary. The abdominal muscles and skin are then closed with sutures. Monofilament sutures such as nylon have been shown to cause less inflammatory reaction in Xenopus skin. Skin sutures should be removed 10 to 14 days postoperatively. Single housing or small group housing for several days (3-5 days) after surgery should be considered as part of the post surgical care of animals undergoing laparotomy. Frogs should be monitored at a minimum daily during this period for appetite as well as for any complications such as dehiscence or infection. Such adverse effects would be reasons for immediate euthanasia.

The IACUC recommends that a maximum of 4 surgeries be performed on each frog. These should consist of three survival surgeries and one non-survival surgery. Investigators must scientifically justify performing more than 4 surgeries on one animal. There should be a minimum of two weeks between surgeries. A record must be kept for each frog. The date that each surgical procedure is performed and the date the animal is euthanized should be recorded.

Additional Information on Hibernation and Cold Storage:

Frogs: Species that naturally hibernate may be held in cold storage if obtained during their natural hibernative seasons. Check with the animal vendor regarding the particular hibernative seasons for the species in question, as well as specific conditions. It is generally recommended that *R. catesbeiana* NOT be placed in cold storage. Hibernating frogs must be kept in dechlorinated water deep enough to cover the animals. The water must be changed at least weekly, or a slow recycling system may be used. Avoid sudden changes in temperature or light

intensity. Temperature should be monitored and documented, and should not fluctuate more than 2-4°C.

Additional Information on Holding Densities:

Fish: It is almost impossible to provide stocking rates, even with regard to a particular proposal, because of the differences in sizes of fish and sizes of aquaria. A density of more than 1.5 cm of fish per liter of water should not be exceeded, unless justification for higher densities is part of the experimental design and documented in a specific IACUC approved animal use protocol. Guidelines from the Zebrafish International Resource Center are as follows: Starting with 20 eggs (1 to 10 h old) or embryos (10 to 72 h old) per 100 ml water, 20 fish can be kept in 400 ml as young larvae (3 to 30 d old); this volume is increased to 3 L as they approach juvenile stage (1 to 4 mo old). Recommended density for growing juvenile fish and holding adults is 5 fish per liter of water. The goal is to achieve 80% to 95% survival with the length of the fish measuring 1.0 to 1.5 cm by 21 d post fertilization. Overcrowding increases stress, susceptibility to disease, and injury as well as the amount of oxygen required in the system for both fish and biological filters. Laboratories that maintain higher than recommended densities of zebrafish should increase the frequency of water-quality monitoring to verify that the system can handle this additional load.

Frogs: Housing density will vary depending on the species of frog. For Xenopus: The density of the tanks should not exceed 4 females or 6 males per 10 gallons of water. It is preferable to house less than four animals per pan (particularly if all four are females, which tend to be much larger than adult males). Animals must be segregated by size to prevent cannibalism. They should be segregated by sex unless breeding is desired and is part of the protocol.

Additional Information on Euthanasia:

Zebrafish embryos, larvae, and adults:
The Zebrafish International Resource Center has completed studies comparing methods of euthanasia for zebrafish embryos, larvae, and adults. It is now recommend that euthanasia of zebrafish embryos, larvae, and adults be achieved via hypothermal shock. Since zebrafish are subtropical poikilotherms, this method is fast (compared to MS222) and effective.

In recent studies, MS222 was found to be an ineffective method of euthanasia for embryos and larvae unless employed for extended periods of time, and fewer signs of distress were observed in zebrafish euthanized by rapid cooling versus MS222; *JAALAS*, 48(6):785-789, 2009.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2786934/?tool=pubmed

A Potential SOP for Euthanizing Fish:

1. Prepare ice bucket every morning (5 parts ice/1 part fish water)
2. Form a depression in the ice to expose water. (For euthanasia of small numbers of fish for fixation or diagnostics use a crossing case, filling the cage with ice, then putting the insert on top of the ice and adding water. This creates a pool of ice water and traps all ice under the insert.)

3. Rapidly, with minimal water, transfer fish (embryos, larvae, or adults) into the ice water depression.

Note: Steps 4-8 apply to maintaining a daily euthanasia system

4. Ice is added throughout the day to maintain an ice slurry 0-4^{o} C
5. There should be a "last call" to euthanize fish in the late afternoon.
6. Before leaving for the night, the person in charge adds bleach to the container to a final concentration of 500 ppm, pH 7. This ensures embryos and larvae are euthanized. It also maintains biosecurity standards and prevents growth of bacteria, fungus, and parasites.
7. The next morning, collect dead fish and dispose of according to local regulations.
8. Prepare fresh ice slush for the day.

AVMA Guidelines for Euthanasia: Freshwater fish and Frogs

Agent	Concentration	Induction time (min)	Route	Comments
MS 222®[1] Finquel®, tricaine methanesulfonate	250mg/L water	>10min*	Immersion, injection into lymph spaces or pleuroperitoneal cavities	*Leave in solution for 10 min following cessation of opercular movement. Large fish may be removed from the water, gill cover lifted and concentrated solution can be flushed over gills. MS-222 is acidic and in concentrations ≥5oomg/L should be buffered with sodium bicarbonate.
Benzocaine hydrochloride	≥250mg/L water	>10min*	Immersion	*Leave in solution for 10 min following cessation of opercular movement Benzocaine alone is not water soluble and therefore is prepared as a stock solution (100g/L), using acetone or ethanol, which may be irritating to fish tissues.
2-phenoxyethanol	0.3-0.4ml/L water(fish) 0.5-0.6ml/L water (frogs)	> 10min*	Immersion	*Leave in solution for 10 min. following cessation of opercular movement.
Sodium	60-100 mg/kg	30 min	IV, Intraabdominally,	Frogs/Toads- may also inject

Pentobarbital		variable	Intrapleuroperitoneally	in subcutaneous lymph spaces. Other barbiturates can cause pain on injection.
CO_2	200ppm		Inhalation	Frogs/Toads- Increased exposure times may be necessary due to their ability to tolerate anoxia.
Inhalant agents	Dissolved 0.5-2ml/l water, or may be bubbled through anesthetic chamber to effect		Inhalation	
Physical methods			Decapitation followed by pithing Note: Xenopus must be double pithed due to atlanto-occipital junction anatomy	May use ultra short acting barbiturate to anesthetize prior to procedure.