Ben Gurion University of the Negev
Preclinical Research Center

Standard operating Procedures (SOP)
For
The Care and Use of Aquatic Animals in Research

November 2014

According to the guidelines of AVMA on Euthanasia, AFS, CCPA, RSPCA

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A. General Information

A-1 Background

Understanding the differences between aquatic and other vertebrate animals, especially mammals, is critically important to the conduct of scientifically valid research on aquatic vertebrates.
Two major aspects to consider:

- Mortality patterns among fish differ greatly from those of mammals, especially in early stages. Thousands or tens of thousands, of eggs and early life stages may produce only a few adult animals. Thus, research on fish, especially field research or research on early life stages normally requires much larger numbers of research subjects than research on mammals.
- Holding and handling requirements for aquatic animals are fundamentally different from the requirements for terrestrial species. Specifically, stable water quality is a key component in housing aquatic species.

The refinement of all aspects of the husbandry, care and use of laboratory animals is important for many reasons: legal, ethical, scientific and welfare. The latter two are inextricably linked, it is increasingly being demonstrated that good animal welfare is essential to produce valid scientific data.

This SOP was developed to ensure appropriate care and use of experimental aquatic vertebrates. The SOP is intended to provide general recommendations to laboratory activities, such as sampling, holding, and handling aquatic species; provide information on administrative matters, including regulations and permits; and address ethical concerns such as pain perceptions or discomfort that may be experienced by aquatic subjects.

A-2 Approval of the Research Project

- Application for use of vertebrate animals in research, testing or teaching. The principal investigators must submit a request to perform the project to the Ben-Gurion University Committee for the Ethical Care and Use of Animals in Research. The application form is found in the Israeli Ministry of Health website under submitting a request for research. Only approved projects can take place. (https://animal.health.gov.il/baaley_haim/e5rev/).

- The housing system for aquatic species must be approved by the attending veterinarian before initiation of the project. When the principal investigator's staff are caring for the animals they must be familiar with the species natural history, physiological needs and provide environmental conditions essential for its well-being. The research team should monitor and recognize changes in environmental conditions and signs of ill health.

- Principal investigators, and the research teams must all be authorized to work with aquatic species through the BGU course 470.2.0100 “Care and Use of animals in research / aquatic species” or an equivalent course recognized by the Israeli Council for Animal Experimentation. For information about the next course please contact the committee secretary Ms. Miri Ben David Ben Yosef ext. 624574, email miribdby@bgu.ac.il .

A-3 Health, Safety and Security

- Researchers should be familiarized with the safety regulations of the institution.
- The principal investigators are responsible for and must comply with, occupational health and safety regulations regarding the protection of personnel from known or suspected physical, chemical, radiation and biological hazards.
- Investigators should be aware of the potential risks associated with zoonotic agents present in aquatic species.

A-3.1 Health of Personnel

- All personal should use rubber gloves and safety glasses when working with noxious chemical used for treatment, cleaning and disinfecting tanks and equipment (e.g. bleach, formaldehyde).
- For handling aquatic species or tank water personnel must don nitrile or other powder free gloves and wash hands with disinfectant soap afterwards.

A-3.2 Safety

- All electrical systems used in aquatic life support systems must be installed and maintained according to the Israeli Electricity Law and manufacturer instructions.
- Dangerous species should be handled in a manner that is safe for both the investigator and the animal being handled.

A-3.3 Security

- All aquatic animals' facilities should have restricted locked access.
- Visitors must be supervised at all times.
- Aquatic species are attuned to their environment, it is important that tranquility be maintained by minimizing physical disturbances.

A-4 Approved Sources of Animals

- **All aquatic animal introductions must be approved by the BGU veterinarians prior to making arrangements to procure them.**
- Approved vendors should be licensed or registered commercial fish suppliers. The fish origin must be from a controlled establishment which is under regular

veterinary inspection.

- For importing fish from other countries please contact the BGU veterinarian for instructions and assistance (ext. 79925 or 79906).
- Generally, an international source must be free of Spring Viremia of Carp (SVC) or other viral diseases for at least two years. The farm that the fish originate from has to have no water connections with other farms affected by viruses. The establishment must be cleared of sanitary regulation problems and have not been prohibited to sell fish. The fish origin must be from a farm were there where no clinical signs of disease when the consignment of fish is conditioned for shipment.

B. Stocking Density

B-1 Fish

- Fish vary from species to species and even within species, as to the degree of crowding that they will tolerate before their behavioral patterns are disrupted. No specific guidelines can be provided, but the potential effects of crowding should be included in each research design.
- The ideal environment for maintaining a given species will have to be developed using performance-based criteria such as growth rate, physiological stress, susceptibility to disease agents, and transmission of disease agents.
- The density with which fish can be held in an experimental unit depends on a series of environmental factors and also the behavioral characteristics of the species. The most immediate concern is maintaining a supply of dissolved oxygen that is appropriate for the species and the temperature of the water. Accumulation of waste products, especially ammonia, is generally the next factor limiting density (Piper *et al*. 1982).
- Sex and age may influence husbandry/care, *e.g.* sexually mature fish may fight and/or adults may eat young.

Standard preferred density for fish:
Ammonia levels "0" OR no greater density than at least two body lengths apart in all directions.

B-2 Aquatic Amphibians

- Population density is one of the most important factors affecting the growth of African clawed frog (*Xenopus laevis*). Frogs with more area available per animal grow significantly faster than animals kept in a tank with a higher population density (Hilken et al, 1995).

- High population density (along with insufficient water levels) affects oocyte quality in females by causing ovary regression (Alexander & Bellerby, 1938).
- Group housing of these frogs in relatively stable groups is advisable.
- Grouping animals can help reduce fear response.
- It is important that animals kept together in the same tank are of similar size.

Standard preferred density for Aquatic Amphibians:
No greater density than at least two body lengths apart in all directions.
A water depth of at least 20 cm is recommended for housing adult *Xenopus laevis* frogs.

C. Transport and Quarantine

C-1 Transport

- Transport of aquatic animals should be in a manner that does not expose them to temperature extremes, low dissolved oxygen or overcrowding.
- All aquatic species exhibit stress when handled and transported.
- Journey times should be the minimum practicable.
- Transport itinerary and conditions, breeding history, dietary background and health status should pass to the establishment receiving the animals before shipment.

C-1.1 In campus:

- Aquatic animals should be transported in a leak proof container, covered with their tank water at all times.
- Avoid exposure to extreme temperatures, to direct sunlight and to public.

C- 1.2 Longer transports:

- Animals which arrive in ill health and do not have a chance to recover, should be euthanized immediately and the sender of the frogs should be informed.
- All cases of mortality within 14 days of arrival from an international source must be reported to the veterinarian and investigated by a pathologist.

C-1.3 Fish

- Methods of handling fish vary with the species and the environment in which they are found (Avault 1996).
- Avoid rough handling, rapid temperature changes, and sudden changes in water

quality, abrasion, and excessively tight confinement.
- Maintaining acceptable levels of dissolved oxygen, carbon dioxide, temperature, ammonia, and pH during transport is essential.

C-1.3.1 Preconditioning Treatments:

- *Addition of salt:* NaCl at 0.5-1% to the transport water to prevent or reduce osmoregulatory dysfunction (Carmichael et al. 1984b).
- *Withhold Feed:* 1-2 days (up to 5 days) prior to transport so there digestive tract is voided and less organic matter accumulates in their shipping water (Weirich 1997).
- *Dissolved oxygen:* The amount of oxygen that can dissolve in the water depends on water temperature; saturation is higher in cool water than in warm water.
- *Proper equipment for transport:* Transport tanks should be well constructed and should be disinfected before use (Avault 1996).

C-1.3.2 Recovery:

- Fish should be allowed to recover in the same or similar medium used for transport (Carmichael et al. 1984b, Weirich 1997).
- The minimum length of time for recovery may vary; generally it is 72 hours following extensive handling.

C-1.4 Aquatic Amphibians

Frogs are captive bred or wild-caught and transportation to the place of use may have to endure a long journey, often to another country or continent. Long distance flights should not pose serious dangers to *Xenopus laevis* and in most instances these animals seem to travel well and appear healthy on arrival.

C-1.4.1 Transport Temperature:

Exposure to excessive heat is the biggest problem for *Xenopus laevis* frogs in transit. Even a few hours at temperatures above 25ºC can damage egg quality and cause ill health in the females (Sive *et al*., 2000).

C-1.4.2 Packing:

- Primary transport box made of water resistant cardboard or polystyrene, and packed with damp sphagnum moss (e.g. NASCO) or damp foam cubes (e.g. Xenopus Express, Xenopus One), into which the frogs can burrow.
- Primary transport boxes placed together in a larger container are usually then surrounded by protective polystyrene insulation packing chips.

- Transport containers must have an adequate air supply and kept in acceptable temperature limits. **Avoid desiccation!**

C-1.4.3 Compatibility:

Frogs being transported together in the same box should be at a similar stage of development, size and weight, and should not be overcrowded.

C-1.4.4 Recovery:

- An appropriately trained and competent person must check the health and welfare of frogs on arrival.
- To prevent potential spread of disease or bacteria, the sphagnum moss and foam cubes in which they were transported should be disposed. (University of California, 2000).

C-2 Quarantine

C-2.1Fish

- Facilities and plans should be in place to ensure that the introduced fish can be isolated physically from fish already present.
- Each holding unit should have its own set of nets and other equipment.
- Facilities and equipment used for previous studies should be disinfected prior to use in new studies, typically with a chlorinated disinfectant.
- The level of quarantine required will vary with the seriousness of the known, or suspected, disease agent.

C-2.2 Aquatic Amphibians

- All new amphibians should be quarantined for 14 days post-arrival.
- Wild-caught amphibians, whether obtained directly or indirectly, should be held for an extended quarantine of 30- 90 days or more due to lifecycles of many of the major parasites of *Xenopus laevis* which are around four to six weeks (Sanders, 2004a).
- During quarantine frogs should be examined daily for signs of ill health, with regard to activity level, skin discoloration, and ulceration, petechial (pinpoint) hemorrhages on the legs, coelomic swelling or any other unusual changes.
- Frogs should be given prophylactic treatments for pathogens during this time.

- Routine husbandry procedures for frogs in quarantine should be undertaken after the resident colony has been attended to and must be carried out with separate dedicated equipment.

C-2.3 Treatment in quarantine

C-2.3.1 General Considerations

- Separation between equipment of the colony housed aquatic animals and the quarantine facility should be kept at all times.
- Separation between lots of aquatic animals is recommended to decrease stress which can bring out diseases and increase shedding of parasites.

C-2.3.2 Low risk or Short-term Holding Period

- Up to 3 weeks in length or using certified clean animals, a minimum of 72 h quarantine is required.
- Perform prophylactic treatment for parasites or if animals are sick.
- Separate nets and pay attention to avoid cross contamination.

C-2.3.3 High risk or long-term Holding Period

- A formal quarantine of 30-60 days depending on risk; should be warranted for animals arriving from uncertified sources.
- Treatments are rendered during this period (according to veterinary inspection and diagnostic of sick fish and anti-parasitic treatment as prophylactic).
- Limiting the access to the quarantine facility (isolated housing facility) separation of nets, separate water supplies.

C-2.3.4 Parasite Control Protocols:

Praziquantel

- 2-3 ppm 24 h bath (can be performed in the original fish tank; dose not harm bio filters)
- The powder should first be dissolved in 70% ethanol on a stirrer until solution is clear.
- Add a small amount of water to the Praziquantel and ethanol solution and continue to stir until solution is homogenized.
- Add the solution to the tank to be treated.
- Monitor the animals hourly in the first 8 hours for sign of stress, stop treatment earlier as needed.
- After 24 h 50% water change should be performed each day for 3 days after

treatment.
- Monitor daily the water quality parameters.
- Repeat the treatment after 10-14 days.

Formalidehyde

- 15-25 ppm 8 h bath with aeration (preferably without bio filters; Formalin can destroy bio filters)
- Monitor fish carefully during the first 3-4 h (if any distress is shown transfer fish to fresh water tank immediately).
- After 8 h 70-90% water change should be performed.
- Monitor daily the water quality parameters.
- Treatment can be repeated every 3 days.

Salt

For freshwater fish:

- 30 ppt dip for 0.5–10 min.
- Carefully monitor fish and if fish show signs of distress (rolling on their side) they should be removed from the bath.
- 2–3 ppt in a freshwater system can minimize parasitic protozoa.

For marine fish:

- 16–18 ppt can be very useful against some parasites.
- Carefully monitoring the fish is essential.

D. Animal Welfare

D-1 General Considerations and Ethical Concerns

- Research involving living aquatic species, must be based on experimental designs that can lead to scientifically valid results, while also taking the welfare of the test animals into consideration.
- Data derived from healthy animals behaving in “normal” fashions is considered representative of “normal” biological function.
- Researchers need to take great care to avoid inducing stress in experimental subjects (especially on a prolonged basis) because it can evoke physiological and behavioral changes (Barton and Iwama 1991).
- Care must be taken to avoid subjecting aquatic species to physically damaging (nociceptive) stimuli because they can evoke stress and/or aberrations in skin and muscle tissue.

D-2 Stress

Stress: “A physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of insult” (Schreck *et al*., 2001).

The set of environmental conditions should best suit the well-being of each species. When aquatic species are maintained, within these ranges, a state of homeostatic balance is achieved. Deviations from homeostasis elicit a stress response.

E. Identification

E-1 General Principles

The following criteria should be applied as much as possible:

- Marking should be quick and easy to apply.
- Marking code (number or colors) should be readily distinguishable.
- Markings should persist on animals until all research objectives are fulfilled.
- Animals should experience no long-term adverse effects on health, behavior, longevity or social life.
- Accurate records of the marking procedure should be kept.
- Marking must allow for seasonal changes and growth of juvenile animals.
- Where such effects are unknown, a pilot study should be implemented.

E-2 Fish

- The identification of fish over time is sometimes required for studies focusing on ecology, fish behavior, age, mortality rates, abundance, population dynamics, migrations, stock identification, and stocking success (Wydoski and Emery 1983, Buckley and Blankenship 1990).
- Researchers can use both intrinsic and extrinsic identification systems, where the nature of the study dictates the type of tag or mark employed.

E- 2.1 Methods for Identification

- The responsibility of the investigator is to ensure that appropriate identification of fish is by the aquaria.
- Each aquarium/tank or set of aquaria housing the same species of fish displays a solid, water resistant identification card or label.
- Individual identification by color, body markings or by using separate aquaria for a small number of individuals which can be distinguished from one another.

E-3 Aquatic Amphibians

Identification of *xenopus* frogs for oocyte harvesting is necessary to make sure the frogs have enough time to recover between surgeries. It is important that only the

most humane methods are used. When considering the acceptability of potential methods it is important to bear in mind that **pain perception in amphibians is likely to be analogous to that in mammals** (CCAC, 2004; Green, 2003).

E-3.1 Methods for Identification

E-3.1.1 Noninvasive Methods:

- Record cards firmly secured on the front of tanks,
- Photographs for recognizing individuals by body markings.

These are good for groups of animals and small number of individuals.

E-3.1.2 Invasive Methods:

- Microchip transponders implanted in the lymphatic cavity (WSAVA 1999) or injected subcutaneously in the area behind the neck are increasingly being suggested for use in amphibians and reptiles (Schaeffer 1999). Frogs normally have to be removed from the water to allow scanning.
- Sewing colored plastic beads onto the muscle mass of the leg or back or attaching studs or tags to the web of the feet. This is to be done in anaesthetized frogs there are no reported perceived health or welfare complications from beads and tags sewing. It could be hazardous to the animal if the thread or beads/tags get caught by the hind claws of that or other animals as they move around (Verhoeff-De-Fremery & Vervoordeldonk, 1982). It could disrupt the mucous membrane of the skin and increase the chance of opportunistic pathogenic infection.

E-3.1.3 Non-approved methods are:

- Applying acetic acid to the skin.
- Leg ringing.
- Removal of toes or fingers.

F. Housing

F-1 Facility

- Regular scheduled preventive maintenance program is needed for all life support systems. Also annual maintenance programs for all other equipment and for surfaces are required.

- The day-to-day operation of the facility, such as scheduled sanitation measures, feeding schedules, and environmental and fish health checks, should be conducted in a standard fashion.
- Facilities should be kept in a clean and orderly manner.
- Tanks should be disinfected before and after every experiment.
- Facilities used for aquatic animal pathogen research must be properly contained and physically separate from other holding rooms and facility functions, such as the holding and rearing of production fish or holding of broodstock fish.
- Effluent must be rendered noninfectious before being returned to the environment.
- Materials and surfaces used for facility construction should be durable, nonporous and easily sanitized using surface cleaning and potent surface disinfectants that have proven efficacy.
- Wood, porous materials and unsealed concrete should not be used.
- Foot baths and hand wash stations should be available at the entry and exit points of the containment facility.
- Room surfaces, piping, tanks and water transfer systems in rooms should be designed for complete access and sterilization between studies, and must be designed to prevent back flow from animal holding tanks and effluent handling systems.
- Equipment for cleaning and sanitation, dry moist feed storage bins and equipment used in the room (such as nets) should be room specific.

F-2 Fish Tanks

- Aquatic environments should be designed to meet the established physical and behavioral requirements of the fish in terms of shelter, social grouping, overhead cover and lighting.
- Tank design should take into account species natural history. E.g., for bottom dwellers, the surface area of the tank is more important than the volume or depth of water. Pilagic fish (that occupy the entire water column) depend more on the volume of water in the tank. Fish that maintain their position by orienting themselves to a directional current or flow require oval or round tanks, while species that need considerable space for swimming prefer an oblong tank. The depth of the tank can also be important as some species will not feed in shallow tanks. Smaller species, such as zebra fish are commonly housed in rectangular plastic tanks.
- Tanks should have smooth, inert, sealed interior surfaces.
- Tanks should be self-cleaning, or adequate means for the regular cleaning. Retention of wastes in fish tanks promotes the proliferation of pathogenic bacteria, protozoa and fungi, and leads to oxygen depletion.
- Tanks should be covered to prevent fish from jumping from the tank, e.g., tank nets or rigid coverings.

F-3 Aquatic Amphibians Tanks

- Tanks should be constructed from material that is smooth, impervious and easy to clean, without sharp edges.
- Plastics used must not add toxicants to the water; acceptable plastics include those used for the storage of human food.
- Tanks with darkened or opaque sides better approximate pond conditions (Sive et al, 2000) and is also likely to be less stressful for the frogs. Both young and adult frogs show preference for dark background coloring (Goldin, 1992) and that a black background coloring may be better for the frogs' wellbeing than a grey or white one (Hilken et al 1995).
- A well-fitting wire or nylon mesh cover can be used to prevent animals from jumping out of the tank. It will also prevent objects accidentally falling into it.
- Tanks should never be completely covered with a solid cover as this will affect air quality and may possibly lead to hypoxia in the frogs.
- Frogs must be provided with refuges to hide in or under (Kreger, 2002; CCAC, 1984; Schaeffer 1999). Frogs are a prey species, therefore it is highly important to provide them with a form of shelter to retreat and hide in.
- Cleaning strategies should be designed to minimize disturbance and distress to the frogs.

G. Husbandry

G-1 General

- Each facility should prepare a facility Standard Operating Procedures (SOPs) manual for acceptable fish husbandry practices and standards.
- All personnel should have working knowledge of the systems that provide clean, tempered water, filtration, lighting and aeration to the tanks and aquaria.
- No one other than the trained worker should adjust these systems.
- Risk and management procedures should be planed for every laboratory/facility and specific guidelines should be followed.
- Record-keeping and documentation, detailed standard operating procedures should be developed for the maintenance and care of all fish and for sanitation of tanks, rooms and equipment.
- Each tank should be monitored **daily** for condition cleanness and animals' wellbeing.
- A log sheet must be completed **daily** (including weekends).
- Cleaning strategies should be designed to minimize disturbance and distress to the animals.
- Disinfectants should be used with extreme caution.

G-1.1 Records:

The following records should be maintained on all fish tanks or in the holding area:

- Animal-use protocol number
- Name of principal investigator
- Emergency contacts
- Source of fish
- Date of arrival
- Species and sex (if identifiable)
- Estimated age / weight
- Daily monitoring sheet

G-1.2 Approved Disinfectants and Sanitation Methods

- Any disinfectant approved for use in aquaculture.
- 1% Bleach solution: for cleaning rooms, empty aquarium and tanks.

G-2 Fish

Daily Log should include:

- Feeding
- Filtration and flow
- Temperature
- Dissolved O_2
- Water PH
- Nitrogen levels (ammonia/ nitrite/ nitrate)
- Mortalities, exams or diagnostic and therapies
- Cleaning tanks
- Cleaning rooms
- Filters change/cleaning

Any out of range values should be documented and treatment corrections should be documented with follow up testing to show corrective measures were effective.

G-3 Aquatic Amphibians

Daily Log should include:

- Diet/feeding schedule
- Water quality (PH, Temperature)
- Mortalities, ill health
- Frequency of cleaning tanks and rooms
- Water changing

H. Water Quality

- Establishing appropriate water quality for each aquatic species is fundamental to maintain healthy animals.
- Each investigator is responsible for determining the preferred conditions for the species under study according to the natural history of the species.
- Contingency plans should be made in case of system breakdown or other emergency, including spare pumps, oxygen delivery systems, heaters etc.

H-1 Water Quality Parameters

H-1.1 Temperature

- Temperature must be kept constant and as in the natural habitat of the species ± 2^{o}C.
- Sudden changes of 5^{o} C in water temperatures can cause serious stress responses in aquatic species that are otherwise healthy.

H-1.2 Dissolved Oxygen

- Sufficient dissolved oxygen is mandatory to fish and many other aquatic species, drop in the required levels will cause immediate death.
- The level of dissolved oxygen required depends on the number of animals in the tank.

H-1.3 Chlorine

- Toxic substances in water such as chlorine (especially free chlorine), from water source can cause immediate death to fish and ill health in other aquatic species.
- De-chlorinating agents, such as activated charcoal filters, and chemicals will reduce free chlorine to undetectable concentrations.

H-1.4 Waste Toxins

- Ammonia, Nitrite and Nitrate are waste products of feed and excreta.
- In closed circuit life support systems waste products are indicative of chronic water quality problems or failure of the bio-filter.
- Elevated nitrate levels may not cause obvious aberrant reactions but can seriously affect the physiology of the fish and research results.

H-1.4 pH

- pH level for most aquatic species is between 6-8 depending on the natural habitat. Minor changes in pH have great effect on the wellbeing of aquatic species.

- The most important factors that alter the pH are biological filtration, respiration and plant metabolism.
- pH usually drops with nitrification (ammonia to nitrate) and respiration (release of CO_2) and rises with denitrification (nitrates to free nitrogen).
- When pH is altered, especially acidic buffering is required.

H-1.5 Alkalinity

- Measuring the buffering or acid neutralizing capacity of water; measured in carbonate hardness.
- Important to keep track of alkalinity especially in closed recirculating systems to prevent pH swings.

H-1.6 Salinity

- Evaporation of water can lead to ion build up over time; measuring NaCl is important if salt is added routinely or if water is added to compensate for evaporation.

H-1.7 Hardness

Calcium and magnesium that remain after boiling of water are parameters of water hardness. There are kits available to measure GH (total hardness) for fish and amphibians.

- 1-4 dGH-very soft water
- 4-8 dGH- soft water
- 8-12 dGH- medium hard water
- 12-18 dGH- fairly hard water
- 18-30 dGH- hard water
- >30-very hard

For aquatic species water hardness depends on the origin of animal. In *X. laevis* the best water hardness is medium to hard water.

H-2 Fish

- Prior to introducing fish, water supplies used to hold fish should be analyzed in detail for parameters, such as hardness, alkalinity (buffering capacity), Na, K, Ca, Mg, CO_3, Cl, HCO_3, SO_4, heavy metals, and pesticides.
- Routine monitoring of temperature, dissolved oxygen, ammonia, alkalinity, nitrite, nitrate and pH should be conducted.

H-2.1 Water quality criteria for optimum fish health; cold fresh water fish, warm fresh water fish and marine species

Parameter	Cold fresh	Warm fresh	Marine	Monitoring frequency		Comments
				Recirculating	Open flow	
Temperature	9-15°c	20-32 °c	Species-specific	Daily	Daily	
Oxygen (mg/l)	7-saturation (>80%)	5-saturation (>80%)	5.5-saturation (>80%)	Daily	Daily	If density is above 15kg/m^3check more frequent
PH	6.5-8	7.5-9	7.5-8.5	Daily	weekly	If density is above 15kg/m^3check more frequent
Ammonia (mg/l)	0.0.0125	0-0.02	0-0.0125	Weekly	weekly	If systems have bio filters check daily during start-up
Nitrate (mg/l)	0-3	0-3	Species-specific	Weekly	weekly	If systems have bio filters check daily during start-up
Nitrite (mg/l)	0-0.2	0-0.1	0-0.2	Weekly	weekly	If systems have bio filters check daily during start-up
Chlorine(mg/l)	0-0.01	0-0.01	Not applicable	annually	Daily (when using municipal water)	Water sources which use chlorine as a disinfectant- municipal water
Total hardness (C_aCO_3) (mg/l)	20-450	50-450	>125mg/l	weekly	weekly	Usually measured by calcium and magnesium but changes in total hardness can relate to changes in alkalinity and PH
Total Alkalinity (mg/l)	10-450	50-450	>150mg/l	weekly	weekly	Recirculation causes a reduction in alkalinity and may reduce PH
Nitrogen (gas saturation)	>100%	>100%	>100%			Check during any fish health problems
Salinity(g/l)	0.1-3	0.1-3	28-35ppt	weekly	weekly	

Adapted from Plumb (1999) and Fisher (2000)

H-3 Aquatic Amphibians

- Water should be completely de-chlorinated before use (Sive et al, 2000) as chlorine attacks the protective mucous layer over the skin and can predispose frogs to infections (Wolfensohn & Lloyd, 1998). This can be achieved by exposure to air for several days in standing tubs.
- A potential danger to frog health and welfare is that a higher pH increases the toxicity of chemicals, such as ammonia, in the water. Even without ammonia present, it is believed a change of 1pH unit (e.g. from 6.5 to 7.5) can lead to the loss of the frogs' protective mucous, a higher susceptibility to pathogen attack, and other stress-related conditions (Sive et al, 2000).
- Water temperature should not be maintained below 16ºC or above 24ºC, with strong consideration given to providing a relatively controlled temperature at a point between 18 and 22ºC.

H-3.1 Water quality standards for *Xenopus laevis*:

Parameter	**Value**	**Monitoring Frequency**	**Comments**
Temperature	18-22^{o}C	Daily	Avoid abrupt fluctuations
PH	6.5-8.5	Daily	
Hardness	75-150 mg/l	Weekly	Not done with carbon filter
Alkalinity (CaCO3)	> 50 mg/l	Weekly	Not done with carbon filter
Salinity	0.4 mg/l (ppt)	Weekly	Not done with carbon filter
Conductivity	50 -2000 µS	Monthly	Not done with carbon filter
Ammonia (NH3)	< 0.02 mg/l	Weekly	Not done with nonrecirculating system
Nitrite (NO2)	< 1 mg/l	Weekly	Not done with nonrecirculating system
Nitrate (NO3)	< 50 mg/l	Weekly	Not done with nonrecirculating system
Chlorine	0 mg/l	Annually	

H-3.2 Effluents

- The potential effects of these wastewaters of facilities holding aquatic species on the receiving ecosystems must be considered.
- Effluents may be discharged continuously or periodically, may combine with other wastewaters, and may discharge directly to a sewage treatment plant or into other city drainage systems, but ultimately they will move into a public water body.

I. Lighting

I-1 Fish

- Light influences, either directly or indirectly, almost all physiological and behavioral processes in fish, including growth and development.
- A 12:12h light cycle is generally recommended.
- Light should be phased on and off, and should incorporate wavelengths and intensities appropriate for the species where this is known.
- Having lights on automated dimmer controls that allow the lights to be gradually brought up in intensity over several minutes is important (Stickney, 1994; DeTolla *et al*., 1995).

I-2 Aquatic Amphibians

- Frogs should not be subjected to bright light, though illumination in the animal house must be of an adequate level to allow observation of the animals and for routine housing and husbandry procedures to be carried out.
- Frogs should have access to places where they can avoid light (Xenopus Express, 2003) as the stress caused by inappropriate lighting will decrease where hiding refuges are provided.
- Employing an artificial sources and block out all outdoor lighting with 14-hour light and 10-hour dark period has been recommended (e.g.Council of Europe, 2003) as roughly corresponding to nature.
- Many establishments choose to use a constant regime of 12 hours light, 12 hours dark (Major & Wassersug, 1998).
- When frogs are exposed to sunlight-equivalent light levels, proper vitamin D levels and correct calcium/phosphorous balance can be maintained (University of Arizona, 2001) and many researchers believe egg quality improves (Sive et al, 2000).

J. Diet and Feeding

J-1 Fish

- Fish must be fed at appropriate intervals (generally daily) with a nutritionally adequate, properly sized feed. Optimal feeding techniques are essential for good health and well-being, and to prevent the fouling of water with uneaten feed.
- Although most species of adult fish can survive several weeks without food, especially at lower temperatures, they must be provided with food that is acceptable to them and that will provide basic nutritional requirements; within a few days if they are to remain in satisfactory condition as research subjects.
- Feeding to satiation is the normal practice unless the research design requires lesser amounts. Typically formulated feeds are fed at levels ranging from 3% to 8% of the weight of the fish, depending on water temperature, and the species, size, and age of the fish.
- Fish must be observed when fed to determine whether they respond as expected, and whether the ration is sufficient or overfeeding is occurring.
- Medicated feeds must only be used under veterinary prescription and supervision.

J-2 Aquatic Amphibians

- *Xenopus laevis* are carnivorous by nature (Phillips, 1979) and although they will scavenge the carcasses of dead animals, they prefer live food (Green, 2002). They have a voracious appetite and will attack almost anything that passes in front of them (Garvey, 2000). They take whatever organisms can be ingested, with insects the most common prey in adult animals (Tinsley & Kobel, 1996).
- Commercially prepared diets for captive frogs. For example, NASCO produce Frog Brittle®. Unfortunately, difficult to get in Israel.
- Beef liver is another item commonly provided but when given on its own has been implicated as a cause of vitamin A toxicity, metabolic bone disease and other amphibian diseases (University of Arizona, 2001; Wright & Whitaker, 2001). Meat (such as liver or heart) should therefore be mixed with vitamins (Mrozek et al, 1995).
- Maggots and crickets can be provided and are stated to appear to provide welcome stimulation and enrichment for the frogs.
- Uneaten particles of food foul the water and debris must be removed after feeding has taken place.
- Care should be taken to leave a sufficient time interval between feeding and cleaning as frogs may regurgitate food if startled, disturbed, or handled shortly afterwards. Frogs and their surrounding water should remain undisturbed for a 3-5 hour period after feeding. Most facilities feed their frogs between 2 and 5 times a week (Schultz and Dawson, 2003).

J-3 Feed Storage

- Feed should be stored in a dedicated area that is dark or covered, temperature and humidity controlled and vermin free to ensure nutritional quality.
- Feed for immediate use and feed in feeders should be similarly protected. Feed used for daily feeding should be kept in sealed-top containers to protect it from humidity and light, and frequently replaced with feed from storage.
- Recommendation from the manufacture should be followed and expiration date should be posted clearly on the sealed container.
- Live feed must be kept frozen or fresh in good sanitary condition.

K. Health

K-1 Fish

- Fish used for research should be free of any notable disease agents that could lead to a diseased condition (unless it is part of the experimental protocol).
- If a disease condition is part of the experimental design, the potential effects of the pathogen on the research results should be predictable, or constitute a variable that is being tested through the research protocol.
- Institutions housing fish for research, teaching and testing should have access to expertise in fish health, and preferably to a veterinarian with aquatic medicine experience and training.
- Handling procedures should be carried out only by competent individuals using techniques that minimize the potential for injury.
- To avoid transition of infection nets and other equipment that are used between tanks should be disinfected with a solution of household bleach after each use (1 cap of bleach (5% sodium hypochlorite) to 55 liters of water) or other approved disinfectant.

K-1.1 Strategic measures for disease prevention should include:

- Fish health oversight done by fish health professional (usually a veterinarian) responsible for the management of morbidity and mortality problems at the facility.
- A program for the detection and management of disease conditions and water quality problems related to physiological stress.
- Strategic application of disease control measures, such as quarantine, immunization and prophylactic treatments.
- Implement a system of regular monitoring and reporting for health assessment purposes.
- All fish in the facility should be monitored on a daily basis for signs of reluctance to eat; unusual behavior or swim pattern, discoloration of the integument and lesions.

K-1.2 Routine Health Monitoring

- Laboratories housing over 1000 individuals of broodstock fish should have 1 fish for every 200 fish held, sacrificed for histopathology analysis. The analysis will be annual (5 fish per 1000 broodstock fish).
- The histopathology analysis will revile:
  - Zoonotic diseases (*e.g.* Microbacteria)
  - Pathologies and pathogens (parasites *etc.*) that can't be seen in routine examination.

K-1.3 Disease Management Protocol:

- Daily observe all animals in the colony.
- Report clinical signs of ill health or mortality to a BGU veterinarian (ext. 79925 or 79906)
- Perform water quality checks when signs of ill health or mortality appear. Correct problems if found.
- Isolate and remove sick or dead fish.
- If unexpected losses of fish occur staff should immediately take water, food and fish samples for later analysis should they be required.
- Retain Samples of affected fish for diagnostic purposes.
- **Apply treatments based on laboratory testing results and recommendations of the veterinarian.**

K-2 Aquatic Amphibians

- Amphibians are prone to many diseases that are often difficult to treat and control (LASA, 2001). It is therefore far easier to prevent, than treat disease in these frogs (DeNardo, 1995).
- *Xenopus* frogs may carry a range of pathogens without the development of disease, until there is physiological upset caused by additional environmental stressors (Tinsley, 1996). This can occur as a result of stress through overcrowding, improper handling, over-use, or poor water quality (Xenopus Express, 2003).
- Providing the most suitable housing conditions and care is vital for maintaining a healthy frog population (Sive et al, 2000).
- Frogs should be observed daily for indicators of poor health. It is very important to know how a healthy individual animal looks and behaves in order to be able to make a comparative judgment. Such animals should be: calm, with moderately slimy skin and a nice pear shape (Sive et al, 2000).
- Ill looking frogs should not be used for oocyte harvest surgeries, due to risk to the animal and risk of poor oocyte quality.
- **Apply treatments based on laboratory testing results and recommendations of the veterinarian.**

K-2.1 Key Signs of Ill Health in *Xenopus* Frogs:

- Dull skin with subcutaneous haemorrhaging (particularly petechial or ‘pinpoint’ hemorrhages on the ventral surfaces of the legs).
- Failure to feed properly or weight loss.
- Erosion or open cuts, lesions or abrasions or ulcerations of the skin.
- Excessive coelomic cavity distension / swelling ('bloating').
- Changes in activity level e.g. lethargy, postural changes.
- Skin discoloration or flaking.
- Diminished avoidance response and righting reflexes.

K-2.2 Behavioral Responses of *Xenopus spp.* Frogs to Aversive Stimuli Include:

- Startle reactions (sudden movements).
- Activity to physically avoid the stimuli (including 'escape' behaviors).
- Wiping or rubbing of the irritated area.

L. Anesthesia

L-1 General

- All methods require that personnel be carefully trained and monitored.
- The environment should, be as quiet as possible.
- Water quality should be similar to that of the environment from which the animal originated, or optimized for that species and situation, for the duration of euthanasia.
- The immersion anesthesia solution should be prepared with water from the animal's housing system.
- Anesthesia methods should be tested in one animal or a small group of animals prior to use in a large population for an unfamiliar species.
- If handling is required, appropriate equipment (nets, gloves) should be used to minimize stressors.

L-2 Fish

L-2.1 Chemical Anesthesia for Fish:

L-2.1.1 Tricaine methanesulfonate, buffered (MS 222, TMS).

- TMS (MS-222) is the most widely used fish anesthetic, and it is extremely effective for rapid induction of deep anesthesia.

- TMS is commonly used in research laboratories and has been registered by Health Canada for veterinary use with fish.
- It is a powder that is easily dissolved in water, with a solubility of 1.25 g/mL water, at 20°C.
- Exposure of a stock solution to sunlight can make it toxic to fish in seawater (Bell, 1987). Because it is an acid, care should be exercised to buffer soft waters with an equal weight of sodium bicarbonate if necessary.
- Dose: anesthetic doses are usually between 25 to 100 mg/L
- Aeration should always be used.
- Induction and recovery times have been shown to be inversely correlated with body weight, with these effects being more pronounced in small fish (Houston & Corlett, 1976).

L-2.1.2 Benzocaine [p-aminobenzoic acid ethyl ester].

- Two forms: a crystalline salt with a water solubility of 0.4 g/L, or a freebase form which must be dissolved in ethyl alcohol first at 0.2 g/mL (Merck and Company, 1989).
- Doses range: 25 to 100 mg/L.
- Induction time is generally in less than 4 minutes and when fish are placed in clean water, recovery is usually within 10 minutes.
- Fish may retain some locomotory functions throughout all stages of anesthesia, making this an unsuitable anesthetic for use in procedures involving surgery.

L-2.1.3 Clove oil and derivatives

- Clove oil has recently been suggested as an alternative fish anesthetic.
- Clove oil is a pale yellow liquid derived from the leaves, buds and stem of the clove tree (*Eugenia sp*.).
- Its active ingredients are eugenol (4-allyl-2-methoxyphenol) and iso-eugenol (4-propenyl-2- methoxyphenol).
- Clove oil is most effective as an anesthetic in fish.
- Dose: 17-20 mg/L for small fish up to 40-60 mg/L for large fish.

L-3 Aquatic Amphibians

L-3.1 Physiological Considerations During Aquatic Amphibian Anesthesia:

- Amphibian anatomy and physiology gives rise to some special considerations in relation to anaesthetizing *Xenopus* frogs. *Xenopus laevis* frogs respire through both the lungs and the skin. They usually rely on lung respiration for the majority of normal gaseous exchange, but this can be affected during periods of anesthesia.

It is therefore essential to ensure that cutaneous respiration (i.e. through the skin) is not also interrupted.

- The animal should be kept moist whilst under anesthesia (Johnson, 1991) by placing wet tissue in contact with the skin (Council of Europe, 2004).
- Fasting of frogs prior to anesthesia is recommended as frogs who have been disturbed may regurgitate food, which fouls the water and increases the chance of inhalation of foreign material while the animal is being anaesthetized (Smith and Stump, 2000).
- Since amphibians are ectotherms, the environmental temperature during anesthesia will affect the frog's metabolism. This, in turn, influences the rate of absorption and excretion of the anesthetic agent and its subsequent effectiveness.
- Successful anesthesia in *Xenopus laevis* is judged by loss of righting reflexes and respiratory effort with subsequent slowing and halting of gular (throat) movements.
- The length of time required for recovery from anesthesia depends on the anesthetic agent, frog's life stage, environmental temperature and depth of anesthesia.
- Frogs should be propped on wet tissue or foam rubber to prevent drowning during recovery (LASA 2001). They should not be returned to their tank until completely recovered.

L-2.2 Chemical Anesthesia for Aquatic Amphibians:

L-2.2.1 Tricaine methanesulfonate, buffered (MS 222, TMS).

- MS 222 is the most common and convenient anesthetic used for *Xenopus laevis* (Halliday, 1999; CCAC, 2004); administered by immersion (300-500 mg/L water) or injection (50-150mg/kg) (Wolfensohn & Lloyd (2003).
- In immersion, induction should take around 5 minutes, though others suggest it could up to 20-30 minutes (LASA 2001).
- MS-222 is acidic when dissolved in water, and so it should be buffered with 50ml of 0.5 M NaHCO3/L (sodium bicarbonate) to a pH of between 7 and 8 (O'Rourke 2002).
- A frog should recover from MS-222 within 15 to 30 minutes after being rinsed in clean water (Wolfensohn & Lloyd 2003). Most establishments are content with the efficacy of immersion in MS-222.

L-2.3 Non Approved Method for Aquatic Amphibian Anesthesia

L-2.3.1 Hypothermia

Hypothermia is not supported by available published articles as a clinically efficacious method of anesthesia in this species (Martin 1995). As there are other, more appropriate protocols, hypothermia should never be used for this purpose.

M. Analgesia

M-1 Fish

Fish respond to noxious stimuli, they have opiate-like hormones and opioid receptors. This implies an endogenous opioid system that might be manipulated to provide analgesia in fish.

M-1.1 Butorphanol

- Opiate agonist antagonist.
- Dose: 0.4mg/kg IM every 24 h
- 1-3 days

M-1.2 Morphine

- Opioid agonist that results in analgesia
- Dose: 5mg/kg IM/IP every 24 h
- 1-3 days

M-2 Aquatic Amphibians

Amphibians nociception pathway has been identified anatomically and histochemicaly.

M-2.1 Flunixin meglumine

- Nonsteroidal- anti-inflammatory drug (NSAID).
- Dose: 25 mg/kg intracoelomic provided good analgesia for 4- 9 h.

M-2.2 Xylazine hydrochloride

- α2 adrenergic agonist.
- Dose: 10 mg/kg intracoelomic provides analgesia for 12-24 h.

M-2.3 Morphine sulfate

- Opioid
- Dose: 40-114 mg/kg in the dorsal lymph sac provides analgesia for 5 h.

N. Euthanasia

N-1 General

Euthanasia - a **"good" or gentle death** - is as important for aquatic animals as it is for other animals.

- All vertebrate animals used in research, testing and teaching must be euthanized in methods approved or conditionally approved by the **AVMA Guidelines for the Euthanasia of Animals: 2013 Edition**
- Euthanasia choices for aquatic species must account for animal stress responses and human safety concerns associated with handling, as well as differences in metabolism, respiration, and tolerance to cerebral hypoxia.

N-1.1 Animal Welfare Considerations

- Loss of consciousness must ensue as rapidly as possible.
- Death, following loss of consciousness, must be as rapid as possible.
- Death must occur without causing pain.
- The method must be reliable and the animal must not regain consciousness.
- There must be minimal psychological stress to personnel and any observers.
- The method utilized should be simple to carry out, with little room for error.
- The environment should be as quiet as possible.
- Water quality should be similar to that of the environment from which the animal originated, or optimized for that species and situation, for the duration of euthanasia.
- The immersion euthanasia solution should be prepared with water from the animal's housing system.
- Euthanasia methods should be tested in one animal or a small group of animals prior to use in a large population for an unfamiliar species.
- If handling is required, appropriate equipment (nets, gloves) should be used to minimize stressors.

N-1.2 Operator's Wellbeing and Research Considerations

- The method utilized must be safe for personnel carrying out the procedure.
- The method utilized must be compatible with the requirements of the experiment.
- All methods require that personnel be carefully trained and monitored.
- Light intensity should be reduced if possible, but with adequate lighting for personnel.

- The method utilized must be compatible with any requirement to carry out further studies on the tissues.

N-1.3 Reliable indicators of death:

- Loss of movement.
- Loss of reactivity to any stimulus and initial flaccidity.
- Respiratory arrest (cessation of rhythmic opercular activity or buccal movement) for a minimum of 10 minutes.
- Loss of righting reflex.

N-2 Fish

- Common methods used to euthanize fish include immersion, injection and physical methods.
- Recommending euthanasia methods for fish and aquatic invertebrates used in biomedical research is challenging due to the enormous number of species and variations in biological and physiologic characteristics.
- If possible, withholding food for 12 to 24 hours prior to euthanasia will reduce regurgitation, defecation, and nitrogenous waste production.

N-2.1 Common Chemical Agents for Fish Euthanasia:

N-2.1.1 Immersion

Overdose in immersion anesthetic solutions is a common method of euthanasia for fish. Fish should be left in the anesthetic solution for a minimum of 10 minutes after cessation of opercular movement.

Benzocaine or benzocaine hydrochloride, buffered. Solutions for immersion should be prepared in concentrations of 250 mg/L and should be buffered.

Carbon dioxide, Immersion in CO2-saturated water causes narcosis and loss of consciousness after several minutes.

Eugenol, isoeugenol and clove oil. Solutions for immersion should be prepared in concentrations of 400-500 mg/L. Products with standardized, known concentrations of essential oils should be used so that accurate dosing is possible.

Tricaine methanesulfonate, buffered (MS 222, TMS). Solutions must be buffered to neutral pH, and concentrations required for euthanasia may vary depending upon the species, life stage, and water chemistry parameters. A concentration of 250 to 500 mg/L, or 5 to 10 times the anesthetic dosage, is effective for most species.

Fish which are too large for practical or cost-effective immersion in lethal doses of buffered MS 222 can be euthanized by applying the concentrated, buffered solution directly to the gills.

N-2.1.2 Injection

Pentobarbital (1 step). 60 to 100 mg/kg can be administered by IV, intracardiac, or intracoelomic routes for euthanasia. Pentobarbital may also be administered via intracardiac injection for anesthetized animals as the second step of a 2-step euthanasia procedure. Death usually occurs within 30 minutes.

N-2.2 Common Physical Methods for Fish Euthanasia

The following methods can be applied for euthanasia, providing they are performed using proper equipment by trained personnel who are regularly monitored for proficiency.

N-2.2.1 Decapitation followed by pithing

Rapid severance of the head and brain from the spinal cord, followed by pithing of the brain, will cause rapid death and unconsciousness.

N-2.2.2 Manually applied blunt force trauma (cranial concussion)

Manually applied blunt force trauma (a rapid, accurately placed blow of sufficient energy to the cranium with an appropriate-sized club) can cause immediate unconsciousness and potentially death, but should be followed by pithing to ensure death.

N-2.2.3 Rapid chilling (hypothermic shock)

- This method of euthanasia is not appropriate for temperate, cool, or cold-water–tolerant fish, such as carp, koi, goldfish, or other species that can survive at 4°C and below.
- It is appropriate for small-bodied (3.8-cm-long or smaller) tropical and sub-tropical stenothermic fish, for which the lower lethal temperature range is above 4°C.
- It is acceptable for zebrafish (*D rerio*) to be euthanized by rapid chilling (2° to 4°C) until loss of orientation and operculum movements and subsequent holding times in ice-chilled water. Adult zebrafish should be exposed for a minimum of 10 minutes and fry at least 20 minutes following loss of operculum movement.

N-2.2.4 Physical Methods as a Second Step

Decapitation, pithing, freezing, and other physical methods for inducing death may be used as the second step when fish have been rendered unconscious prior to their application

N-2.3 Unacceptable Methods for Euthanasia of Fish

- Flushing of fish into sewer, septic, or other types of outflow systems.
- Slow chilling or freezing of unanesthetized animals, including placing fish into a freezer without prior anesthesia.
- Anoxia after removal from the water or by anoxia in water.
- Exposure to caustic chemicals.
- Prolonged traumatic injury prior to unconsciousness.

N-3 Aquatic Amphibians

- Amphibian species commonly used in research include the African clawed frog (*Xenopus laevis.*) and leopard and bull (*Rana spp.*) frogs. These species are best euthanized via a physical method while fully anesthetized.
- Brain activity can persist for significant periods of time in amphibians, even though they may show no behavioral responses
- The animals may be suffering if they are held in aversive chemical agents or have undergone physical damage.

N-3.1 Injection methods

N-3.1.1 Tricaine methane sulfonate (MS 222)

Into the dorsal lymph sacs or intracoelomically, 200-300mg/kg of a 1% buffered solution (Stanford University 2003).

N-3.1.2 Sodium pentobarbital

Into the dorsal lymph sacs or intracoelomically, 60-100 mg/kg, (Sanders 2004(b), CCAC 2004).

N-3.2 Immersion methods

Potential stress for an animal (i.e. through handling and injection) can be reduced if drugs are administered by dissolving them in the water in which the amphibians are placed (European Commission 1997) NB. The agent is absorbed into the animal through the skin.

N-3.2.1 Tricaine methane sulfonate (MS 222)

- Use a concentration of >0.3% (3g/liter) (Schultz 2003) or 0.2% solution should be used for at least three hours (Halliday 2003, LASA 2001).
- MS 222 must be buffered to near pH 7 with sodium bicarbonate, to prevent the frog suffering pain or irritation from this agent (which lowers pH),
- As the immersion time needed to assure death can range from 20 minutes to three hours, many researchers may use MS222 as an anesthetic followed by a physical method of euthanasia (Stanford University 2003).

N-3.2.2 Benzocaine hydrochloride

Use a concentration >250mg/L (for at least 10 mins) has been suggested.

N-3.3 physical Methods

N-3.3.1 Concussion

- Concussion can be an effective and humane way of stunning amphibians if carried out by a person who is well trained in the method (European Commission 1997).
- Concussion of the brain by striking the cranium, with the destruction of the brain before the return of consciousness, amphibians, reptiles and fish brains are extremely tolerant to hypoxia.
- It cannot be assumed therefore that the effects of concussion will be irreversible, or that even subsequent decapitation would necessarily destroy brain function in the time to avoid the return of sentience.
- In the case of amphibians, reptiles and fish, if the brain is not destroyed by the initial blow, there must be no delay in destroying the brain by a penetrating probe or by a blow sufficient to cause a severe brain contusion with fracture of the cranial bones.

N-3.4 Unacceptable Methods for the Euthanasia of Amphibians:

N-3.4.1 Decapitation or double-pithing

- If used as a sole method is no longer approved by many ethical committees on welfare grounds.
- Decapitation, by itself, does not produce immediate unconsciousness within the severed heads of amphibians (Stewart, in UFAW 1989) and should therefore only be used if the amphibian has been made unconscious first by other methods (European Commission 1997).
- To ensure brain death, subsequent double-pithing (insertion of a sharp needle through the base of the brain and the spinal cord) while the animal is still unconscious is advised.

N-3.4.2 Hypothermia

- Hypothermia should never be used either for the purposes of anesthesia or euthanasia in amphibians (Schaeffer 1999).
- This is because cooling the body does not reduce the animal's ability to feel pain (ANZCCART 2001).
- Cooling followed by freezing of frogs for euthanasia could also cause suffering due to ice crystal formation, both on the skin and within the body (European Commission 1997).

N- 3.4.3 Hyperthermia

Hyperthermia is slow and painful, so is inhumane.

N-3.4.3 Formaldehyde solution

Amphibians, even in larval form, should not be killed either by placing them directly in formaldehyde solution (University of Arizona 2001).

N-3.4.4 Exsanguination

Exsanguination may not render amphibians immediately unconscious, which causes suffering and distress.

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