EUTHANASIA OF FINFISH, INVERTEBRATES, & BIRDS

PERFORMANCE STANDARD: When required for scientific data collection, clinical, or operational requirements, animals will be humanely killed, as detailed in the approved protocol or according to this policy.

BACKGROUND: This policy covers only finfish, invertebrates, & birds. There are separate polices for ‘rodents,’ ‘non-rodent mammals,’ and ‘amphibians & reptiles.’ All species of animals used at Duke are to be euthanized in a humane manner which minimizes distress and maximizes a smooth and event-free death. Unintended recovery of animals after euthanasia constitutes serious non-compliance with PHS Policy and serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals. Duke is obligated to report such non-compliance to the NIH.

Although the perception of pain requires a conscious experience, defining consciousness, and therefore the ability to perceive pain, across many species is quite difficult. Previously it was thought that finfish and invertebrates lacked the anatomic structures necessary to perceive pain as we understand it in birds and mammals. While some invertebrate taxa include animals with no nervous system (e.g., sponges) and nervous systems with no ganglionation or minimal ganglionation (e.g., starfish), other invertebrate taxa have well-developed brains and/or complex behaviors that include the ability to analyze and respond to complex environmental cues (e.g., octopus, squid, cuttlefish). Most invertebrates do respond to noxious stimuli and many have endogenous opioids. The Duke IACUC has determined that invertebrates belonging to the PHYLUM Mollusca, CLASS Cephalopoda (e.g., octopus and squid) are covered species for animal care and use oversight and management, including euthanasia.

The Animal Care Unit of the Animal and Plant Health Inspection Service, U.S. Department of Agriculture; the NIH Office of Laboratory Animal Welfare (OLAW); and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) have issued guidance regarding methods and expectations of euthanasia. Publication of the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition has further refined the expectations for euthanasia.

ROLES:
1. Researchers and animal care staff will abide by this policy as described below.
2. Researchers and animal care staff are expected to follow the methods of euthanasia as outlined and approved in their protocols or DLAR/DLC-approved SOPs, unless alternate euthanasia methods are IACUC-approved prior to their performance.
3. This policy outlines the procedures and methods generally considered to be acceptable for the Duke research community. However, other methods may be approved by the Duke IACUC for specific research situations and/or protocols given appropriate scientific justification.

4. All individuals who may perform or be expected to perform euthanasia should be familiar with the details in the protocol and clarifications outlined in this policy. Core training is available by web module; more detailed training in the proper methods of euthanasia is available by contacting DLAR veterinary staff at 919.681.6792.

**PROTECTIVE POSTURES REQUIRED:** Personnel Protective Equipment (PPE) routinely required for normal animal care or use is required for euthanasia activities.

**DEFINITIONS:**

1. **Humane Euthanasia** consists of a primary method and a secondary physical method. Failure to complete both methods results in failed humane euthanasia.

2. **Secondary Methods** consist of physical disruption of tissues or organs. Acceptable secondary methods include exsanguination, harvest of vital organs (such that life is no longer continuing), pithing, or decapitation (with or without pithing). Cervical dislocation is not an acceptable secondary method.

3. **Euthanasia Chambers** may consist of the animal’s home (preferred), such as the cage, box, tank, aquarium; or a container specifically designed for the purpose of euthanasia. The euthanasia chamber must provide adequate exposure to allow unobstructed viewing of the animal during the euthanasia activity for identification of appropriate progressions from an alert state, to an insentient state, to death.

**POLICY:**

1. **Routine Euthanasia:** Any method listed in this policy may be used for humane euthanasia in Duke University animal activities, providing the method is described in the IACUC-approved protocol.

2. **Emergency Euthanasia:** In an emergency, any method listed in this policy may be used to end animal suffering and prevent further animal distress. Such 'off-protocol' activities must be reported to the IACUC as soon as practical, but within 48 hours of performing the euthanasia.
3. **Responsibility:** The PI and all protocol participants are responsible for the welfare of the animals assigned to their protocol(s), including the manner in which the life of the animal(s) is terminated.

4. **‘Musts’ applicable to all euthanasia activities:** The general euthanasia principles which must be addressed in any euthanasia activity are:
   a. Individuals must be qualified in the technique they are performing.
   b. Euthanasia must follow AVMA guidelines, unless a specific exception has been approved by the IACUC.
   c. Euthanasia must follow methods approved in the Duke protocol.
   d. Death must be assured by a secondary physical method.
   e. Personnel qualification must be documented; most commonly using the Personnel Qualifications Form (PQF) of the protocol. Other training records may also be used to document skills.
   f. Live alert animals and dead animals (or deeply sedated animals) must never be placed together.
   g. A secondary method of euthanasia must always be performed. Secondary methods are chosen based upon species variability and life cycle of the animals. Commonly used secondary methods include:
      i. Decapitation (all),
      ii. Pithing (fish, amphibians),
      iii. Collection of vital tissues (e.g. heart, major organs) sufficient to assure the animal will not recover (all), or
      iv. Exsanguination (all).

   **NOTE:** If no secondary method is specified in the protocol, then decapitation is the default and expected procedure for a secondary method of euthanasia.

   **NOTE:** These ‘musts’ are requirements. These must are not suggestions or simply good ideas!

5. Disposal of any carcass or parts (all animals) that are not immediately used requires:
   a. Placing the carcass (or parts) in a bag or container; and
   b. Labeling the bag/container with the date of euthanasia, the PIs name, and the person who performed the euthanasia.

   **NOTE:** If animals are to be disposed of by DLAR, bags compatible with the tissue digester must be used.

6. **General Considerations:** Recommending euthanasia methods for finfish, invertebrates, or birds used in biomedical research is challenging due to the enormous number of species and variations in biological and physiologic characteristics. Methods for euthanizing species are expected to be described in the research protocol.
a. **Finfish:**

i. **General Considerations:**

1. **MS 222:** Available as tricaine methane sulfonate (TMS), MS 222 can be used for the euthanasia of amphibians and fish. Tricaine is a benzoic acid derivative and generally should be buffered with sodium bicarbonate. A 10 g/L stock solution can be made, and sodium bicarbonate added to saturation, resulting in a pH between 7.0 and 7.5 for the solution. The stock solution should be stored in a dark brown bottle, and refrigerated or frozen if possible. The solution should be replaced monthly and any time a brown color is observed.

2. **Secondary Methods of Euthanasia:** Death must be assured by a secondary method of euthanasia. Secondary methods for these animals include:
   a. Decapitation,
   b. Pithing,
   c. Collection of vital tissues (e.g. heart, major organs) sufficient to assure the animal will not recover, or
   d. Exsanguination.

   **NOTE:** If no method is specified in the protocol, then decapitation is the default and expected procedure for a secondary method of euthanasia.

ii. **Laboratory-managed aquatic species other than zebrafish/medaka:**

1. **MS222:**

   a. Fish are placed in a solution containing ≥250 mg/L MS222. Fish should be left in this solution for at least 10 minutes following cessation of opercular movement. Large fish may be removed from the water, a gill cover lifted, and a concentrated solution from a syringe flushed over the gills.

iii. **Zebrafish/Medaka (≥ 8 DPF (days post-fertilization)):** Options include:

   1. **Hypothermia in chilled water:** Fish are immobilized by submersion in chilled water (2-4°C). Fish are maintained in the chilled water for at least 10 minutes following cessation of opercular (i.e., gill) movement. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 20 minutes after cessation of all movement to ensure death by hypoxia;
2. **MS222**: Fish are placed in a MS222 solution containing ≥250 mg/L. Fish should be left in this solution for at least 10 minutes following cessation of opercular movement.
   a. Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen; or
   b. Decapitation with a sharp blade by a trained individual when its use is required by the experimental design and approved by the Institutional Animal Care and Use Committee.

iv. **Zebrafish embryos: 4-7 DPF**: Options include:

   1. **Hypothermia in chilled water**: Immobilization by submersion in ice water (2-4°C) for at least 20 minutes to ensure death by hypoxia.

   2. **Hypochlorite**: Addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. They should remain in this solution at least five minutes prior to disposal to ensure death.

   **NOTE**: Pain perception has not developed at these earlier stages so this is not considered a painful procedure (see references).

v. **Zebrafish embryos ≤ 3 DPF**: Development should be terminated using bleach.

   1. **Hypochlorite**: Addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. They should remain in this solution at least five minutes prior to disposal to ensure death.

   **NOTE**: Pain perception has not developed at these earlier stages so this is not considered a painful procedure (see references).

vi. **Pond or mesocosm (outside areas) containment (Gambesia, etc.)**: Fieldwork on finfish may be conducted on a smaller scale under conditions that make euthanasia feasible. In such cases, convenience for the researcher should not be a primary consideration.

   1. **Acceptable methods** for field work, include:

      a. **Immersion**: Immersion in solutions of buffered tricaine methanesulfonate (MS 222), buffered benzocaine, quinaldine sulfate, isoflurane or sevoflurane, and 2-phenoxyethanol.
b. **Injection:** An injection of pentobarbital (60 to 100 mg/kg) can be administered IV or intracoelomically. Pentobarbital may also be administered intracardially in anesthetized animals. A two-step injection procedure is encouraged. Approaches include:
   1) Ketamine (IM) followed by a lethal dose of pentobarbital;
   2) A combination of ketamine and medetomidine (IM) followed by a lethal dose of pentobarbital; and
   3) Propofol (IV) followed by a lethal dose of pentobarbital.

**NOTE FOR DRUG RESIDUES:** Although a general concern for all environments and situations, the potential effects of drug residues and proper disposal of animal remains should be considered when using any of these drugs.

2. The following methods are acceptable with conditions for use in this environment:

a. **Immersion** in CO2-saturated water or eugenol, isoeugenol, or clove oil; followed by pithing.

b. **Decapitation followed by pithing.** Decapitation alone is not considered a humane form of euthanasia, especially for species that may be particularly tolerant of low O2 concentrations. Pithing helps ensure rapid death for those species.

c. **Cervical transection followed by pithing.** The rationale for this approach is similar to that for decapitation and pithing, except that the head is still physically attached by musculature to the body.

d. **Rapid chilling** (hypothermic shock) in water of 2° to 4°C for small-bodied (3.8-cm-long or smaller) tropical and subtropical stenothermic species (as previously described for zebrafish). Because of surface to volume considerations, use of this method is not appropriate in medium to large-bodied finfish until pertinent data for those species becomes available.

vii. **Verification of death of finfish:** Methods used to verify death after completion of the primary and secondary methods include observation of gill movement, auscultation, ECG, or Doppler ultrasound. It is important to remember that hearts may beat even after brain death. Other methods may be considered by the IACUC for certain locations and uses.
NOTES FOR FINFISH:
1. These methods ensure death provided the timeframes above are followed.
2. The ice water methods should not be extrapolated to other aquatic species without first confirming the effectiveness for that species. Aquatic species native to a colder environment (non-tropical fish) may be more resistant to hypothermic shock and may subsequently recover; an event requiring a regulatory report.
3. Current OLAW interpretation of PHS policy considers aquatic species as "live vertebrate animals" at hatching. Although this is an imprecise stage for zebrafish, it can be approximated at 72 hours post fertilization.
4. Do not allow fish to come in contact with ice (ice will cause localized freezing of tissues which is painful and must not be permitted).

b. Invertebrates: Overdose of a general anesthetic is an appropriate euthanasia strategy for aquatic invertebrates, and immersion an effective route of administration. Because confirming the death of many invertebrates is difficult, a 2-Step euthanasia methodology is required in which chemical induction of anesthesia (e.g., producing nonresponsiveness and/or presumptive death) is followed by an adjunctive physical method that destroys the brain or major ganglia physically (e.g., pithing, freezing, boiling) or chemically (e.g., alcohol, formalin). Application of the latter methods as a sole method is not acceptable.

i. First-Step Agents:
1. Magnesium salts are a near-universal anesthetic agent, relaxing agent, and euthanasia agent for aquatic invertebrates. A range of concentrations has been recommended for various phyla. Research suggests the magnesium ion acts centrally in suppressing neural activity of cephalopods.
2. Ethanol has been used for euthanasia of some phyla (at a 1% to 5% concentration as compared with concentrations of > 70% used for preservation), and acts by inhibiting neuronal sodium and calcium channels in molluscs. Initial aversion and/or excitement has been reported as occurring in cephalopods.
3. It is acceptable for invertebrates (tropical and subtropical stenothermic species) to be euthanized by rapid chilling (2° to 4°C) until loss of orientation and movements and subsequent holding times in ice-chilled water. Adult animals should be exposed for a minimum of 10 minutes.
ii. Second-Step Agents:

1. Noninhaled agents that can be administered via immersion as the second step of a 2-step euthanasia approach include 70% alcohol and neutral-buffered 10% formalin. These agents are not acceptable, however, for immersion as a single-step procedure, nor as the first step of a 2-step procedure.

2. Physical methods include pithing, freezing, and boiling are acceptable as the second step (adjunctive methods) of a 2-step euthanasia procedure. Pithing requires detailed anatomic knowledge of the species in question.

   NOTE: These methods are not acceptable as a single-step procedure, nor are these methods to be used as the first step of a 2-step procedure.

iii. Verification of death: Methods used to verify death include auscultation, observation of respiration / movement of gills or muscles. It is important to remember that hearts may beat even after brain death.

c. Birds:

   i. Primary Methods: While many methods and practices common to other species may be used in birds, the acceptable methods are:

   1. Intravenous barbiturate or barbiturate combinations
   2. Overdose of inhaled anesthetics (generally isoflurane)
   3. Decapitation (small birds)
   4. Carbon Dioxide (CO₂) Inhalation
   5. Thoracic Compression

   a. Euthanasia using CO₂: Since the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition require that animals are placed in an atmosphere of 100% room air and transitioned to 100% CO₂ over several minutes, there are certain requirements when using a designated CO₂ chamber. The chamber must be completely flushed with room air for 1-2 minutes between each euthanasia event to dislodge captured CO₂ from the container. Smaller chambers may be turned on their side for 1-2 minutes between each euthanasia event. Turning the chamber on its side will allow an opportunity for the pooled CO₂ to drain from the chamber. Since CO₂ is heavier than air, cages must first be “dumped” to facilitate flushing. Diffusion is too slow in the absence of dumping. In addition, the chamber must also be sanitized after
each use. Leaving fecal material, bedding, or other refuse in the euthanasia chamber is not good sanitation.

NOTE: Failing to complete either of these actions is a noncompliance with the ‘Euthanasia Policy,’ and may have an impact on continued approval of CO₂ as a means for euthanasia.

1) **Methodology for humane CO₂ euthanasia:**
   - **A transparent chamber must be used.** Place the animals in a transparent chamber. If you cannot clearly observe the animals in the chamber, then the chamber is not acceptable. The purpose for observing the animal(s) progressing to euthanasia is to assure the transition is smooth and uneventful. Restless or excited animals may be experiencing dysfunctional CO₂ delivery and the process should be modified to accomplish humane CO₂ euthanasia.
   - **The chamber must not be overcrowded.** Every animal in the chamber must be able to place its feet on the floor of the chamber at the same time, with enough additional space so that when it does become unconscious, it will lie down on the floor of the cage and not on top of another animal.
   - **Species must not be mixed** when performing euthanasia. If you must euthanize several species at the same time, you must use separate chambers. Rodent species (e.g., rats and mice) may be predator or prey depending on the context. Predation stress is not humane and counter to the principles of humane euthanasia.
   - **Euthanasia in the home cage is the preferred method.** Animals mixed immediately prior to euthanasia may be distressed as they try to re-establish social order. Social distress is counter to the principles of humane euthanasia. The preferred approach is use of the home cage for euthanasia; this minimizes distress associated with the event. If cages of the same species are mixed they should be euthanased as soon as possible after mixing.
   - **The chamber must be filled gradually.** Place the animals in the chamber before initiating the flow of CO₂. The overarching guideline for filling the chamber is provision of a CO₂ flow between 20% and 30% of the chamber volume. **DO NOT EXCEED A FLOW RATE OF 30%.**

The formula for the flow rate calculation is:
- \[ \text{Volume (in L)} = \frac{{\text{(height in cm) x (width in cm) x (length in cm)}}}{1000} \]
- Maximum acceptable flow rate = (Cage Volume in Liters) X 0.3
2) **The IACUC must document proper euthanasia.** Failure to have a flow meter in the CO₂ set-up, failure to post the calculated maximum flow rate or failure to stay below the calculated maximum flow rate will be regarded as non-compliance! While the goal is to provide the calculated 30% flow rate, if you cannot be on target, be below. Example: Calculated rate is 3.85L/min. the flow meter only shows 0.5 L increments; therefore provide 3.5 L/min. **NOTE:** Exceeding the calculated flow rate is non-compliance with both the AVMA euthanasia guidelines and with our institutional agreements with NIH.

3) **There must be sufficient time in a CO₂ atmosphere to ensure death.** The Duke position is that the time required in a CO₂ atmosphere for euthanasia is from the point where cessation of breathing has been observed, plus an additional 2 minutes. Using these guidelines breathing will generally stop between 4-5 minutes and the heart will stop beating at between 5-6 minutes after beginning the procedure. For safety, wait an additional 2 minutes after cessation of breathing (a total of 6-7 minutes) to assure the animal is dead. If fresh tissue is required for laboratory tests (e.g., fresh pancreas for RNA analysis); animals may be removed from the CO₂ chamber following sustained cessation of breathing (4-5 min), provided a physical secondary method is performed immediately (e.g., organ removal, major vessel being cut)!

**Thoracic** (cardiopulmonary, cardiac) **compression** is a method used to euthanize wild small mammals and birds, mainly under field conditions. According to the 2013 **AVMA Guidelines**, thoracic compression is an unacceptable means of euthanizing animals that are not deeply anesthetized or insentient due to other reason. The AAALAC Council on Accreditation has recognized the need for the use of thoracic compression in conscious wild small birds in situations where alternate techniques are not feasible or objectives of the protocol are such that the Institutional Animal Care and Use Committee (IACUC) may grant approval for this method, **provided** that training for the technique is completed and its continued approval is re-evaluated as more scientifically-based data regarding its use becomes available.
ii. **Secondary Methods:** Because it is often difficult to confirm death in bird, the application of at least one physically disruptive method is required. Approved secondary methods include:
   1. Decapitation
   2. Removal of organs
   3. Thoracotomy

iii. **Verification of death:** Methods used to verify death after completion of the primary and secondary methods include auscultation, ECG, Doppler ultrasound, or pulse oximetry. It is important to remember that hearts may beat even after brain death. Other methods may be considered by the IACUC for certain locations and uses.

**NOTE:** In cases where decapitation has occurred as the mode of euthanasia, none of these verification methods are required.

**NOTE:** Consult the 2013 AVMA guidelines for additional methods that may be acceptable for specific species in specific circumstances.
References

- AAALAC Council on Accreditation exception to the Guidelines statement
- Guidelines for the Use of Fish in Research
- Guidelines to the Use of Wild Birds in Research