

Zebrafish 101 for IACUCs

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OLAW Online Webinar March 12, 2015

Zebrafish 101 for IACUCs

- Focus of Monte Matthews' presentation
 - Institutional Oversight
 - Training and Education for both IACUCs and Zebrafish Researchers
 - When to Have a Protocol
 - Counting Zebrafish
- Focus of Dr. George Sanders' presentation
 - Occupational Health and Safety
 - Veterinary Care: Anesthesia and Euthanasia

Institutional Oversight: Recommendations for Selecting IACUC Members

- Investigators from departments that use zebrafish
- Senior zebrafish animal care management staff
- Representatives from EH&S, compliance, IACUC staff, grants management
- Non-scientific members: english, philosophy, public relations, legal
- Others: student member, e.g., biology grad student
- Alternates
- More than one vet
- Length of service
- Odd number: easier to obtain quorum
- Voting vs. non-voting members



How to Foster a Team Approach to Zebrafish Oversight

- More than just lip service....
- Include PIs in policy development
 - Husbandry SOPs, IACUC policies
- Inviting new PIs to IACUC meetings
- Have researchers assist with training program
- Asking questions, such as how can we help?
- Making recommendations in semiannual report to the IO



IACUC Training and Education Resources

- Institutional IACUC Member Handbook
- AVMA Guidelines for the Euthanasia of Animals (2013)
- Standard Operating Procedures (e.g., Zebrafish Book)https://wiki.zfin.org/display/prot/ZFIN+Protocol+Wiki
- On-line training for zebrafish or aquatics
 - CITI: Collaborative Institutional Training Initiative https://www.citiprogram.org
 - AALAS Learning Library (ALL)https://www.aalaslearninglibrary.org
- Workshops
 - MDI Biological Laboratory Health and Colony Management of Laboratory Fish: http://mdibl.org/course/hcmlf-2015/
 - Gadsden State Community College and University of Alabama at Birmingham: http://www.gadsdenstate.edu/academics/science/an/zebrafish.php



Zebrafish-specific Training and Education Resources

Many general reference materials for IACUC members:

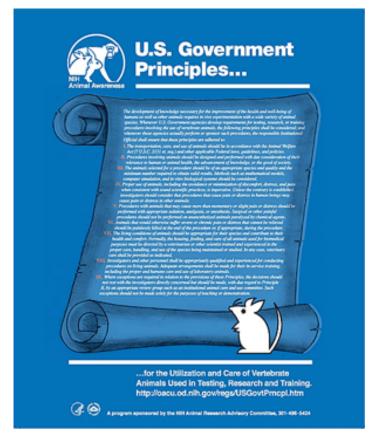


Training and Education

 Incorporate the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training

Basis for ethical discussion

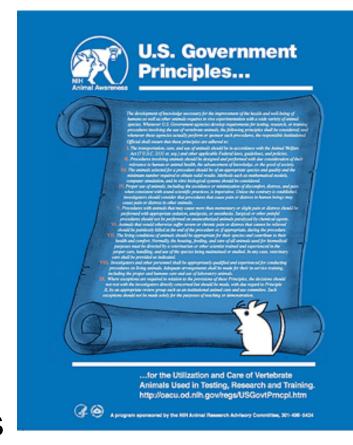
- PHS Policy page 4
- Guide page199



Training and Education: Principle IV

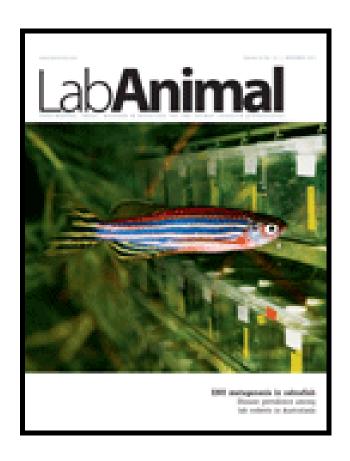
"Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative.

Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals."



Training and Education

Lab Animal Volume 40 (11), Nov. 2011 ENU mutagenesis in zebrafish



Is a Protocol Necessary? Definition of Animals

- PHS Policy: Any **live**, **vertebrate** animal used or intended for use in research, research training, experimentation, or biological testing or for related purposes.
- *Guide:* laboratory animals...defined as any vertebrate animal (e.g., traditional laboratory animals, agricultural animals, wildlife, and **aquatic** animals) produced for or used in research, testing, or teaching.

Is a Protocol Necessary?

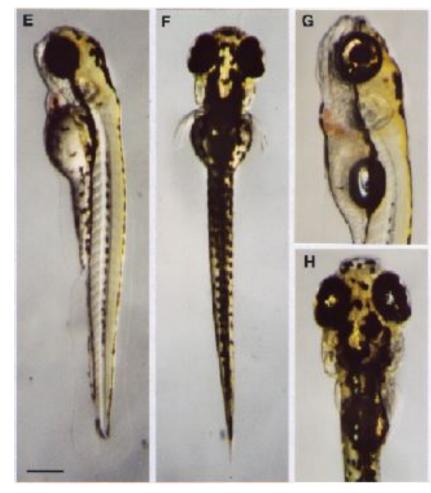
OK, but what about fish embryos and larvae? Larval forms of fish are covered by the PHS Policy.

OLAW guidance FAQ A5 http://grants.nih.gov/grants/olaw/faqs.htm#App_5



Early Larval Stage Characteristics

- Early larvae (3-7 dpf)
 - Almost free swimming
 - Melanophore development
 - Swim bladder inflated at 4-6 days
 - Active feeding (4 dpf)
 - Yolk reserves last up to 5 days
 - Startle response
 - Upright orientation
 - Optokinetic response
- Mid larvae (7-21 dpf)
- Late larvae (21-30 dpf)



5-6 dpf

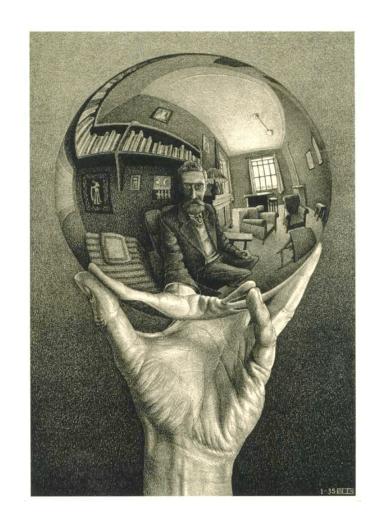
Is a Protocol Necessary?

Reasons UO covers all stages of zebrafish development

- PHS Policy rationale for approximate number
- OLAW guidance on hatching
- Justification of number of adults needed
- Embryos → larvae → juveniles
- Compliance issues
- Legal definition of vertebrate animal
- Care for embryos / early larvae
- IACUC protocol template
- Opportunity for replacement

Is a Protocol Necessary?

If your institution doesn't cover all embryonic stages of development, then your IACUC should have a written policy or SOP to address unexpected hatching to be compliant with OLAW guidance.



Is a Protocol Necessary for Field Studies?



Leech protection

OLAW FAQ A6 http://grants.nih.gov/grants/olaw/faqs.htm#App 6



Is a Protocol Necessary?

What about field studies funded by National Science Foundation?



http://www.nsf.gov/pubs/policydocs/pappguide/nsf15001/gpg 2.jsp#IID7 http://www.nsf.gov/pubs/policydocs/pappguide/nsf15001/aag 6.jsp#VIB3

Is a Protocol Necessary for Field Studies in Foreign Countries?



OLAW FAQ D13 http://grants.nih.gov/grants/olaw/faqs.htm#629



Is a Protocol Necessary for Collaborations?

- No requirement for dual IACUC review
- Formal written agreement and understanding
 - Who is responsible for off-site care of animals?
 - Who owns the animals?
 - Which IACUC has responsibility for oversight and protocol review?
- Institution should have a policy

OLAW FAQ D8 http://grants.nih.gov/grants/olaw/faqs.htm#621



Counting Zebrafish

Do we have to count all those zebrafish?

If so, what flexibility do we have?







Zebrafish 101 for IACUCs

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Zebrafish are NOT Aquatic Mice





Image source:

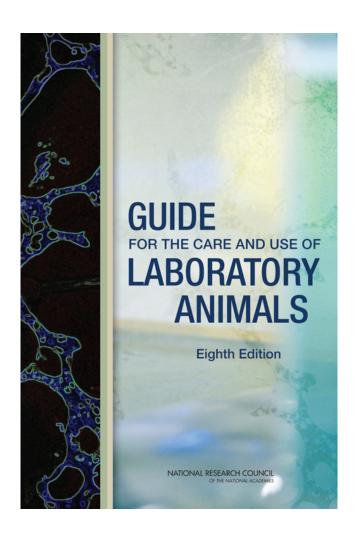
http://upload.wikimedia.org/wikipedia/commons/a/ac/Zebrafisch.jpg

Image source:

http://upload.wikimedia.org/wikipedia/commons/0/0d/%D0%9C%D1%8B%D1%88%D1%8C 2.jpg



Occupational Health and Safety



Chapter Two Page 17 - 23



Personal Protective Equipment (PPE)

- Dedicated outer garments
 - Scrub tops or lab coats
 - Laundered on site
- Gloves (non-powdered)
 - Adequate length
 - Vinyl or nitrile (Gutleb, et al.)
- Frequent hand washing
 - Non-scented soaps
 - Before and after handling fish or husbandry equipment
- Closed toe, non-slip shoes
 - Dedicated shoes
 - Shoe covers
 - Foot baths



Image source:

http://www.wsha.org/files/82/HandWash.PNG

Disposable PPE

- Single-use only so do not reuse
 - Gowns
 - Shoe covers
 - Hair bonnets
 - Face masks
- PPE should be specified by circumstances or completed hazard risk assessment by the occupational health and safety program



Launder Reusable PPE on Site

On a regular schedule clean and disinfect reusable personal protective equipment

- On site: use facility dedicated washing or drying equipment
- Off site: contract work out to commercial clothing service vendor







Additional Important Safety Considerations

- Slips and Falls
 - Standing water on floor or equipment
 - Working on or around ladders
- Electrocution
 - All electrical outlets should be GFCI protected
 - Check equipment for loose or damaged wires, seals, etc.
- Traumatic injury
 - From broken glass or other sharp objects
- Ergonomic related or repetitive movement disorders
 - Protect to prevent Carpal tunnel syndrome
 - Heavy lifting: 1 gallon water = 8.34 pounds
- Chemical exposures
 - Location and use of spill kits



Important Safety Reminder



Photo provided by Dr. George Sanders

Do not re-cap needles!! Re-capping needles is unsafe!



Zoonotic Diseases and Allergies

- Diseases: transmitted between animals and people
 - Bacterial: mycobacteria (acid fast), gram negative/positive
 - Parasitic:
 - Cryptosporidium (protozoan)
 - Pentastomes & Cestodes (helminthic)
 - Fungal: Trichophyton, Candida
- Immune-compromising medical conditions or treatments
- Allergies: sensitivity to fish or shellfish proteins
- Important to be enrolled in the institutional occupational health and safety program
- Discuss any potential medical concerns with your primary care physician before working in a zebrafish facility



Mycobacterium marinum Nguyen, Chau. NEJM 350:9 February 26, 2004



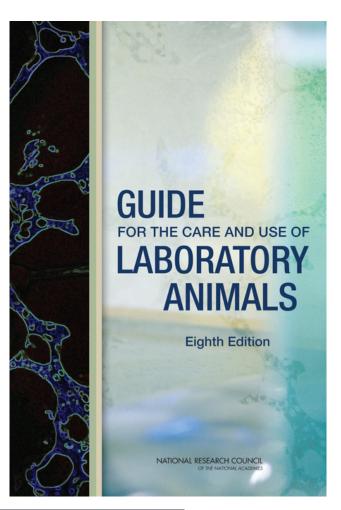
SMALL, RAISED, ERYTHEMATOUS LESION DEVELOPED ON THE DORsum of the hand of a 35-year-old man who worked in a pet shop. Over a period of three months, there was progressive spreading of nodular lesions from his hand to just above his elbow. There was no epitrochlear or axillary adenopathy. The patient did not have fever or other symptoms. His duties included cleaning fish tanks daily, which he did without wearing protective gloves. Needle aspiration of a nodule recovered thick, purulent fluid that was positive for acid-fast bacilli, identified on culture as Mycobacteríum marínum. The patient was treated successfully with a four-month regimen of rifampin and ethambutol.

M. marinum infection is an occupational hazard for aquarium cleaners, fishermen, and seafood handlers. Since growth of the organism requires a low temperature (between 24° and 32°C), infection is usually limited to the skin, with the hands, elbows, and feet most commonly affected. Dissemination of the infection, even among immunocompromised patients, is rare.



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Veterinary Care: Anesthesia and Euthanasia



Chapter Four Page 121 - 123



Anesthesia of Zebrafish

- Minimize movement during procedures that would cause at most momentary pain or distress
 - Examination, weighing, measuring
- For procedures in which movement must be minimized and more than momentary pain or distress would be expected:
 - Fin clipping for genotyping
 - Placement of chips, tags, and colored elastomers for individual identification purposes
 - Manual gamete removal
 - Surgical procedures



Stages of Anesthesia

- Stage I: Swimming erratic, some loss of equilibrium
- Stage II: Obvious loss of equilibrium, swimming slow and aimless
- Stage III:
 - Plane 1: Complete loss of equilibrium, slow swimming and respiration
 - Plane 2: **SURGICAL**: Swimming movements absent, respiration rapid and shallow
 - Plane 3: No respiratory activity but can revive
- Stage IV: Spasmodic flaring distention of the opercula cardiac failure imminent



MS-222 (Tricaine Methanesulfonate) Preparation

- Stock solution: 4 10 mg/ml
 - Do not buffer
 - Typically frozen at 20 C and will be good for 3 months
 - Must label and date
- Working solution: 0.2 mg/ml
 - Buffer solution to pH 7-8 with sodium bicarbonate
 - Typically refrigerated at 4 C and will be good for 1 month after creation
 - Must label and date
- Solutions are light sensitive: must store in dark or light-tight bottle



Anesthetic Administration

- Test water pH to ensure adequate buffering
- Immerse fish in buffered MS-222 solution at 150 mg/L dose in system water
- Fish may be placed on a moist sponge during the procedure
 - Apply fresh anesthetic water over skin and gills
- Recover fish in regular system water
- Other anesthetics can be used similarly e.g., benzocaine hydrochloride



Zebrafish Euthanasia

- AVMA Guidelines for the Euthanasia of Animals 2013 Edition https://www.avma.org/KB/Policies/Documents/euthanasia.pdf
- Guidelines for Use of Zebrafish in the NIH Intramural Research Programhttp://oacu.od.nih.gov/arac/documents/zebrafish.pdf
- Matthews, M. and Varga, Z.M. 2012. Anesthesia and Euthanasia in Zebrafish:
 Institutional Animal Care and Use Committee Considerations. ILAR 53(2): 192-204.
 http://ilarjournal.oxfordjournals.org/content/53/2/192.full.pdf+html
- Blessing, J. J., J. C. Marshall, and S. R. Balcombe. 2010. Humane killing of fishes for scientific research: a comparison of two methods. J Fish Biology 76, 2571–2577.
- Wilson, J.M., R.M. Bunte, and A.J. Carty. 2009. Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (Danio rerio). JAALAS 48 (6): 785–789.
- Strykowski, J.L. and J. M. Schech. 2015. Effectiveness of Recommended Euthanasia Methods in Larval Zebrafish (*Danio rerio*). JAALAS 54 (1) 81-84.



Euthanasia Methods of Adult Zebrafish

- Anesthetic Overdose
 - MS-222 or benzocaine hydrochloride (>200 mg/L) via immersion
 - Buffer euthanasia solution to pH 7-8 with sodium bicarbonate before exposure
 - Wait until 10 min after last opercular movement ends
- Eugenol (clove oil)
- CO₂ (compressed gas cylinder)
- Physical euthanasia methods must be followed by a secondary method to ensure death
 - Cranial concussion and decapitation
 - Decapitation and double pithing
 - May require IACUC approval and certification



Zebrafish Euthanasia: Rapid Chilling

- Tropical fish species, like zebrafish can be euthanized by rapid chilling to between 2 – 4 C
- Rapid chilling protocol
 - Place the fish in a small volume of water in a container of adequate size
 - Fish must not come into direct contact with ice
 - Add four times the volume of ice on top of water
 - Monitor water temperature with a thermometer
 - Keep fish in chilled water until 20 minutes after orientation loss and operculum movement cessation
 - Remove fish and store in freezer until appropriate carcass disposal



Rapid Cooling: Hypothermal Shock

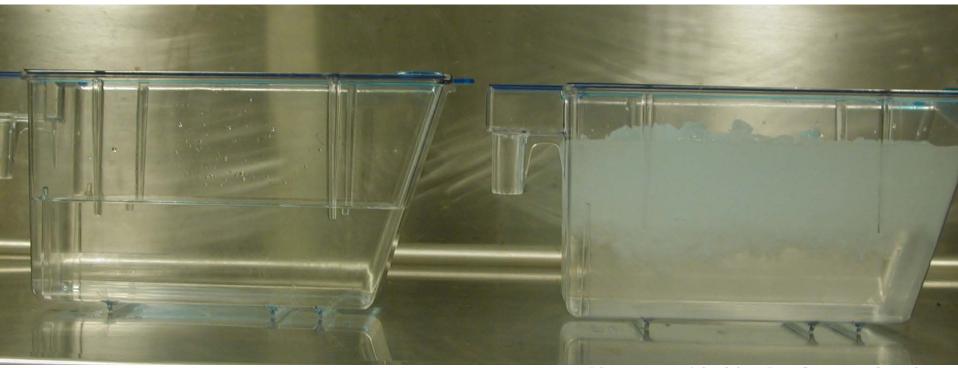


Photo provided by Dr. George Sanders



Euthanasia of Embryos and Larvae

- Euthanasia is difficult to confirm by visual observation
- Requires a longer exposure to immersion anesthetics or ice water
- Requires exposure to higher concentrations of immersion anesthetics
 - Larvae and embryos utilize cutaneous gas exchange for oxygen needs and immersion anesthetics do not affect this process
- Research has shown that exposure to immersion anesthetic or ice water is not reliable for euthanasia of these life stages because animals may recover



Euthanasia of Embryos and Larvae with Bleach

- Embryos and larvae can be euthanized by addition of bleach (sodium hypochlorite) to system water at the rate of 1 part bleach to 5 parts of water
- They should remain in this solution at least 5 minutes to ensure death
- This method is unacceptable as a sole means of euthanasia beyond embryonic and larval stages



Verification of Death after Euthanasia

- Death must be verified after euthanasia to comply with the AVMA Guidelines
- Adjunctive methods should be applied only to unconscious fish with complete cessation of opercular movements, examples:
 - Exsanguination (severing large blood vessels e.g., gill arch or tail vein)
 - Freezing
 - Rapid severance of the head and brain from the spinal cord (decapitation)
 - Maceration using a well-maintained macerator appropriate for the size of the fish



Zebrafish Carcass Disposal

- Carcasses are typically frozen
- On a scheduled basis, carcasses are transferred to medical waste, biohazard disposal, rendering, composting, or sanitary landfill according to facility standard operating procedure



Additional References

- National Research Council. 2011. *Guide for the Care and Use of Laboratory Animals* 8th ed.
- Harper C, Lawrence C. 2011. The Laboratory Zebrafish. Boca Raton: CRC Press.
- Collymore, C. A. Tolwani, C. Lieggi, and S. Rasmussen. 2014. Efficacy and Safety of 5 Anesthetics in Adult Zebrafish (*Danio rerio*). JAALAS 53:(2) 198-203

Zebrafish Husbandry Association Webinar

Dr. George E. Sanders – March 31, 2015 Preparing Your Facility for IACUC Inspections

http://www.zhaonline.org/webinar-series.html

OLAW's FAQ A5 asks, "Does the PHS Policy apply to larval forms of amphibians and fish?

OLAW's answer is, "Yes, larval forms of fish and amphibians have vertebrae and are covered by the PHS Policy. As noted in FAQ A4, the PHS Policy applies to the offspring of egg-laying vertebrates only after hatching. Zebrafish larvae, for example, typically hatch 3 days post fertilization."

Our IACUC would prefer not to oversee the welfare of zebrafish larvae until they are feeding, free swimming fish, at 5 dpf. How do you justify your guidance?



OLAW oversees the welfare of live vertebrates. This applies to egglaying vertebrates after hatching.

Zebrafish hatch at approximately 3 dpf, when the it is no longer protected by its chorion and has developed features defining it as a vertebrate (notochord, neural tube, pharyngeal arches, somites and posterior tail). Therefore, OLAW defines the hatched zebrafish as a vertebrate animal.

There are several ways your IACUC could meet the requirement for oversight of zebrafish larvae. Some institutions cover all live stages of zebrafish in their research protocols. Other institutions have a core protocol that covers larvae and fry until they are transferred to the research protocol at the required stage needed (i.e., post 3 dpf). And then many other institutions have protocols that cover all zebrafish beginning at 3 dpf.

Do Mycobacterium and other microbial agents come from the fish or are they environmental? If they come from the fish, do they also grow in the water or on the sides of the tank?



Mycobacteria and other agents are found in and on the fish and are also in the aquatic environment in which the fish are housed.

As to the second question, yes, these agents also grow in the water and on the side of the tank.



How is decontamination of the tank and water accomplished given the sensitivity of zebrafish to their environment?

Could you briefly discuss tank cleaning?

What about contamination on fish nets and other equipment you use?



Answer 3.1

Zebrafish users must always be hyperaware of anything coming into contact with their zebrafish systems.

Decontamination of tanks can be done by several different methods and is often determined by the capabilities of the facility or area in which the zebrafish are housed.

Tanks can be cleaned with mechanical methods such as dishwashers, cagewashers, or manual gross organic debris removal by hand or with the use of an in-sink glass washer; high heat such as autoclaves; or disinfectants such as bleach, net soak, 1% Virkon, etc.



Answer 3.2

Tank screens, baffles, plugs, and lids can be scrubbed thoroughly to remove any feed detritus and then can be processed in the same manner as the tanks.

Nets can be autoclaved, sanitized in a mechanical washer, and / or bleached (1% solution) followed by soaking in a dechlorination solution. Autoclaving will reduce the useful life of nets; thorough dechlorination is essential before the nets are used.

Any use of chemicals must be carefully considered and used with extreme care.

Answer 3.3

Water is decontaminated with the system's recirculation filtration process as described by Dr. Sanders



Dr. Sanders described the various forms of PPE including the importance of gloves and hand washing. But in Mr. Matthews slides of field studies, the investigator was not wearing PPE or gloves.

Is there a risk to the fish or the investigator? Should PPE be used in the field?



Yes, adequate PPE should be used in the field and gloves are typically worn when working with fish and potentially contaminated equipment. I have another picture of the same investigator wearing appropriate gloves while handling a fish net.



Dr. Sanders advised that the stability of MS-222 is 3 months and recommended storage at -20 C. Is this the stability in the frozen state or after MS-222 has been thawed?



The stability of MS-222 begins to degrade once either the stock or working solution are made. Freezing or refrigeration only slows down the degradation process.



Since there are only two sources of MS-222, can the non-pharmaceutical grade MS-222 be used solely for the purpose of zebrafish euthanasia?



Since pharmaceutical grade MS-222 is available, that is the one that must be used. The use of a non-pharmaceutical grade euthanasia agent must meet the same criteria that OLAW and USDA require for other substances used in animals.

For further guidance, please refer to FAQ F4 at URL http://grants.nih.gov/grants/olaw/faqs.htm#662



Our institution uses pharmaceutical-grade MS-222. Can we use expired pharmaceutical-grade MS-222 for euthanasia?



Expired pharmaceutical grade MS-222 should not be used for euthanasia. Euthanasia, anesthesia and analgesia agents should not be used beyond their expiration date, even if a procedure is terminal.

For further guidance, please refer to FAQ F5 at URL http://grants.nih.gov/grants/olaw/faqs.htm#663



How is respiration observed or measured in sedated zebrafish in water?



Respiration is observed or measured in sedated zebrafish in water by visual observation of the movement of the fish's opercula while under sedation or anesthesia.



Is a garbage disposal an acceptable macerator for the euthanasia of zebrafish?



AVMA guidelines describe a required macerator as a "well maintained macerator designed for the size of finfish being euthanized".

The AVMA guidelines also state that "flushing of finfish into sewer, septic, or other types of outflow system is unacceptable for many reasons."

The development and application of a performance standard would determine whether a garbage disposal proposed for use as a macerator meets the AVMA Euthanasia Guidelines and is, therefore, acceptable to the IACUC.



Since pain management is limited in zebrafish, is it possible to have fish in Column D?

Could technique, use of MS-222, housing, and euthanasia at certain endpoints be considered appropriate measures to relieve pain and distress?

(This institution uses the USDA categories for all animals on their campus even though they understand that fish are not USDA-regulated.)



Many institutions choose to use the USDA pain categories for all species, not just USDA regulated animals. Hence, category D is defined as, "Number of animals upon which experiments, teaching, research, surgery, or tests were conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs were used."

In this example, MS-222 is proposed, as well as a modification in housing, such as keeping them in an area with a lower light intensity and noise level for the purpose of minimization of pain, distress or discomfort. The development of humane endpoints would also be appropriate.

When are adjunct or secondary methods of euthanasia required with zebrafish?



The use of adjunct or secondary methods of euthanasia are required to ensure that zebrafish are dead when death cannot be confirmed by observation.



Is environmental enrichment a consideration for zebrafish?



Yes, according to the *Guide*, the use of environmental enrichment for zebrafish must be considered by the IACUC, investigators, and attending veterinarian.



Comment 13

System design is specifically geared towards rearing the intended species. One would need to know the optimum living parameters (water chemistry; tank dimensions, shape and possibly color; noise, vibration, light intensity) of the animal to be raised and the intended use of the animal.

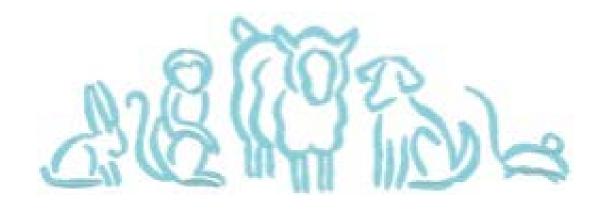
In the case of zebrafish, it would be egg production. In the agricultural fish industry, it would be meat production.

Then, one would build a system to accommodate each. Each system will be unique and operate and be maintained according to the target need. For instance, in zebrafish production, keeping genetic lines isolated is critical, so installing tank anti-jump mechanisms and fine filters to act as fish exclusion devices is necessary.

Comment 14

It is important to understand that when zebrafish get sick, it is likely caused by an environmental issue (e.g., poor water quality, disrupted light cycle) that is causing stress leading to disease.

In the case of an outbreak, although understanding the causative pathogen is important, the top priority should be isolating the environmental change that is causing the stress.



OLAW Online Seminar Topic: Non-pharmaceutical Grade Substances June 4, 2015

