Guidelines for Use of Zebrafish in the NIH Intramural Research Program

General Guidelines

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. For purposes of accountability all stages of development greater than three days of age should be described in an approved Animal Study Proposal. Thus an estimate of the number of larval zebrafish from day 4-7 days post fertilization (dpf) should be included in Animal Requirements (Section B in the NIH ASP Template).

Since these early stages (4-7 dpf) do not to feel pain or distress, it is preferable that their numbers be separated from zebrafish ≥8 dpf. This number can be listed as Column C in the Pain and Distress Category (Section H) of zebrafish ASPs as a separate number from zebrafish ≥8 dpf.

The pain and distress categorization of the ≥8 dpf fish should be determined by the investigator based on the specific procedures described in the protocol. The number of animals used may need to be provided as an estimate, particularly with these young larvae, considering their size and normal housing conditions. Estimated numbers may still be used after they have matured to adults if they are group housed.

Euthanasia Guidelines

Recent observations indicate that zebrafish up to at least 15 dpf can survive anesthetic overdose and rapid chilling even after prolonged absence of heartbeat. They can revive if returned to water that is within their normal environmental parameters. An adjunct method such as sodium hypochlorite treatment should be used to ensure death in embryos <15 dpf.

Similarly, embryos less than 3 dpf that are being disposed should be treated with sodium hypochlorite to prevent further development.

Euthanasia of zebrafish must be carried out by the following methods.

1. For zebrafish ≥15 dpf the following methods are acceptable for euthanasia:
   - Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4º C) 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.
   - Overdose of tricaine methane sulfonate (MS222, 200-300 mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement. MS-222 solution should be buffered with sodium bicarbonate to a neutral pH before immersing fish. Non-buffered MS-222 is acidic and causes an aversive reaction in unanesthetized fish.
   - Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen.

2. For zebrafish larvae up to 8-15 dpf: a secondary method must be used in order to ensure death. Use of the ice water or MS-222 method as above should be used as a method of anesthesia/immobilization. An acceptable secondary method is the addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. The larvae should remain in this solution at least five minutes prior to disposal to ensure death.
3. For embryos ≤ 7 dpf, development should be terminated using bleach as described above. Pain perception has not developed at these earlier stages so this is not considered a painful procedure.
4. Additional methods can be used if approved by the IC Institutional ACUC committee:
    - Clove Oil (Eugenol, Isoeugenol) as an alternative to MS-222. AVMA Guidelines recommend that products with standardized, known concentrations of essential oils (eugenol, isoeugenol) be used so that accurate dosing can occur. Clove oil and eugenol products are described in the AVMA Guidelines as "acceptable agents of euthanasia for finfish." They are not available in an FDA approved form but there is at least one commercial form available in the U.S. (Aqui-S) as an Investigational New Animal Drug.
    - Decapitation with a sharp blade by a trained individual.
    - Anesthetic overdose or rapid chilling by submersion in ice water followed by fixation in paraformaldehyde or other fixative
    - For embryos <8 dpf: immersion in paraformaldehyde or other fixative.
    - For embryos <8 dpf: rapid freezing in -70 freezer. Embryos should be contained in a minimum amount of water to ensure rapid freezing and death.
    - Maceration using a well maintained macerator designed for the size of the fish being euthanized.

Zebrafish carcasses from any of these methods should be disposed of as Medical Pathological Waste according to NIH policies.

These methods ensure death provided the timeframes above are followed. The ice water method should not be extrapolated to other aquatic species without first confirming the effectiveness for that species. Aquatic species, native to a colder environment than zebrafish, may be more resistant to hypothermic shock and may recover subsequently.

References

1. University of Oregon (2008) Final Report to OLAW on Euthanasia of Zebrafish.
2. National Institutes of Health (2009) Final Report to OLAW on Euthanasia of Zebrafish.
3. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition.
4. Matthews M and Varga Z. (2012) Anesthesia and Euthanasia in Zebrafish *ILAR Journal* 53(2):192-204.
5. Wilson JM, Bunte RM, Carty AJ (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*) JAALAS 48:785-789

http://www.ingentaconnect.com/content/aalas/jaalas/2009/00000048/00000006/art00013?token=00581fe75594f6247483ab65a666f3a7b6c53402576316b65217d3f3449264f65263a3d4f58762f46c9748b6

6. Strykowski JL and Schech JM (2015) Effectiveness of recommended euthanasia methods in larval zebrafish (*Danio rerio*). *JAALAS* 54(1): 81-84. http://www.ingentaconnect.com/content/aalas/jaalas/2015/00000054/00000001/art00013?token=00521c2474d0fa192b2c437a63736a6f5e47354c3b663f702c495b6f644a467b4d616d3f4e4b344f9f

Approved 03/11/09
Revised 05/08/13, 06/22/16