

Guidelines for Use of Zebrafish in the NIH Intramural Research Program

Scientific Background

These guidelines are predicated on the need to minimize suffering and distress in zebrafish. Suffering requires that the animal have both the neural apparatus for detecting noxious stimuli as well as the mental ability to interpret such stimuli as aversive (1). Many studies have demonstrated that adult zebrafish show evidence of higher order cognition, being responsive to a variety of learning protocols (e.g. 2, 3, 4, 5), including learning to avoid aversive stimuli (6, 7, 8, 9). Thus while the ability of adult fish to experience suffering remains controversial in the scientific literature [for recent reviews reaching conflicting opinions see (10) and (11)], there is sufficient evidence to take a cautious approach in adult zebrafish by instituting guidelines that ensure rapid euthanasia.

In contrast there is no evidence of higher order cognition in zebrafish during the first week of development. Developmental studies examining learning (12), reward (13), social (14, 15) and fright (16) behaviors have found that these functions become operational only in older fish. During the first week of development, embryonic movements are simple reflexes that do not provide evidence for a capacity for suffering. Thus during the first week, zebrafish larvae can respond to simple stimuli but have not reached the point in brain development where stimuli can be experienced as aversive.

Zebrafish larvae during the first week resemble early mouse embryos in that they are chiefly sustained by nutrients derived from the yolk. The criterion of nutritional independence for developmentally immature animals is subject to empirical verification and has found support in international regulations for the welfare of immature vertebrates (17). While the capacity for suffering is the primary criterion for establishing a threshold for 8 days post fertilization (dpf) for euthanasia in zebrafish, the criterion of independent feeding also supports this age.

Hatching occurs at approximately 72 hours (which would be at the end of day 3 post fertilization), although hatching is not an accepted staging index in zebrafish (18). Zebrafish larvae are not able to feed upon hatching and are chiefly sustained by nutrients derived from yolk which is not depleted until 7 dpf (19). Only after 7 dpf do zebrafish larvae manifest signs of ill health in the absence of external feeding (20). Active feeding can not commence at hatching because brain structures required for detecting and catching prey have not developed. At hatching, larvae lack taste buds (21, 22), have poor visual acuity (14), and cannot swim effectively as they lack a swim bladder and have deficient motor control (23, 24). Therefore in zebrafish the period between hatching and nutritional independence at 8 dpf is essentially an extension of the early embryonic stage during which the fish continues to develop sensory and motor functions required for the independent larval stage.

Thus during the first week of development, zebrafish remain in an immature state consuming yolk for nourishment and responding to stimulation with simple reflexive movements. As larvae become nutritionally independent during the second week of life, they also acquire more sophisticated cognitive abilities.

Euthanasia Guidelines

Euthanasia of zebrafish must be carried out by one of the following methods. Although not described in the AVMA Euthanasia Guidelines, these procedures have been shown effective for euthanizing zebrafish (25).

1. For zebrafish ≥ 8 dpf the following methods are acceptable for euthanasia:

- Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° 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.
- Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen.
- Decapitation with a sharp blade by a trained individual when its use is required by the experimental design and approved by the IC Institutional Animal Care and Use Committee (26).

2. For zebrafish 4-7dpf the following methods are acceptable for euthanasia (27):

- Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° C) for at least 20 minutes to ensure death by hypoxia.
- 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. As detailed above in the scientific background section, pain perception has not developed at these earlier stages so this is not considered a painful procedure.

3. For embryos ≤ 3 dpf, development should be terminated using bleach as described in section B2 above.

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.

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 - 7dpf should be included in Animal Requirements (Section B in the NIH ASP form).

Since these early stages (4-7dpf) do not 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.

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Approved 03/11/2009