HU IACUC RECCOMENDATIONS FOR ZEBRAFISH USE

Public Health Policy on Humane Care and Use of Laboratory Animals Policy

These institutions defines animals as, “any live, vertebrate animal used or intended for use in research, research training, experimentation or biological testing or for related purposes.”[1] Furthermore, the Office of Laboratory Animal Welfare interpretation of PHS policy considers aquatic species as "live, vertebrate animals" at hatching.[2] Although this is an imprecise stage for zebrafish it can be approximated at 72 hours post fertilization.

Guidelines for Animal Study Protocols (ASPs)

A. For purposes of animal number accountability, all stages of development greater than three days post fertilization(dpf) should be counted in an approved ASP. Due to the high throughput nature of the zebrafish model, large numbers of animals may be listed on ASPs. However, the majority of studies are completed on larval zebrafish between 4-7dpf. Therefore, to understand animal use, an Institutional Animal Care and Use Committee (IACUC) may request that investigators report their monthly animal numbers separately as larval stages (4-7 dpf) and those animals reared past > 8dpf. (See Study Proposal Form).

B. When evaluating “Gender as an experimental variable…”, the Principal Investigator (PI) and IACUC must consider age since sex is undetermined until at least 25 dpf. [10,11]

Standard Operating Procedures for Zebrafish Handling

A. Receiving Live Animals

a. Zebrafish are shipped/transported in sealed, water filled, plastic bags with oxygen added. Upon arrival shipping bags are inspected for signs of damage and shipment accuracy.
b. Inspect fish for any signs of stress, trauma, or mortality that may have occurred while in transit.
c. Fish and fish embryos from approved vendors are introduced into an appropriate size tank in the Quarantine room.
d. Fish are acclimated to the aquatic system within 24 hours of arrival by emptying the fish into an empty tank connected to the system.
e. Fish arriving with health concerns, or found sick, are isolated from healthy fish by placing them in their own isolation tank outside the system and the Facility Manager, PI and Clinical Veterinarian notified as needed.
f. Individual shipments are identified by source, date of arrival, and any pertinent information.
g. New tank numbers are recorded at time of arrival.
h. Only bleached embryos are transferred out of Quarantine into the primary housing area.

B. Housing

a. Fish should be housed in the authorized fish tanks. The maximum density is 5 adult fish per liter of water.
b. A net is utilized for catching fish that must be removed from a tank.
c. Water conditions and quality for Zebrafish are maintained as follows:

Tank Water Parameters			
Parameter	**Range**	**Preferred**	**Confirmed**
Temperature	72-82^{0}F	78.^{0}F	Daily
PH	6.8-7.8	7.4	Daily
Conductivity	400-600 µS	550 µS	Daily
Alkalinity	50-200 ppm	170 ppm	Weekly
Hardness	0 dGH	0 dGH	Weekly
Ammonia	0	0	Weekly
Nitrite	0-25 ppm	0	Weekly
Nitrate	0-40 ppm	10 ppm	Weekly

d. Room light cycle, 14 hours light (e.g., 9 AM till 11 PM) /10 hours dark cycle.
e. Housing access is limited to IACUC certified personnel only.

C. Feeding

a. Zebrafish are fed a combination of live feeds and commercial processed dry feeds (e.g., Tecniplast ZEBRAFEED 200-400, ZEBRAFEED 100-200).
b. Zebrafish should be fed twice daily by adding enough food to each tank so that food is available to all fish and nearly all food is eaten within 10 minutes.
c. When feeding brine shrimp, use freshly harvested shrimp. Collect shrimp into a pipette while trying to avoid collecting any unhatched eggs into the pipette.
d. When hand feeding, feed approximately one 1.5 ml pipette of shrimp per 10 adult fish. This can vary depending on the size and number of fish in the tank.
e. Place pipette in the top of the tank and dispense about half the contents into the tank.
f. Wait for the fish to consume most of the shrimp then dispense the remaining shrimp into the tank.
g. Rinse pipette after each use. Place unused shrimp in the refrigerator for future use
h. Record feeding.

D. Daily Duties

a. Conduct daily monitoring of zebrafish morbidity and mortality. i. Removing sick fish from the population is our most effective means of detecting pathogens and adverse environmental conditions, preventing spread of disease, controlling morbidity, and ensuring a high level of fish welfare.

ii. Observations of sick fish and communications must be noted on the Room Status Sheet and the Facility Manager's Animal Health and Environmental Concern Form. Tanks with health concerns are flagged with a red sticker tab.

iii. Remove Fish exhibiting the behavioral and physical signs below

- Fish at surface or near water inlet
- Rapid breathing

- Sluggish movement
- Rubbing on tank surfaces
- Circling or spinning
- Loss of equilibrium
- Fin erosion or skin lesions
- Mass swelling or weight loss
- Exophthalmia
- Hemorrhaging

b. Place dead fish in a cooler at +4°C for 48 hours and lab informed.
c. Feed Zebrafish twice daily as outlined above.
d. Check the tanks and verify systems are operating properly, refer to the tank user manual for more detailed information.
e. Inspect display and record the temperature, pH, and conductivity.
f. Visually inspect the sea salt and bicarbonate tanks (e.g. refill as needed).
g. Inspect the system. Additional maintenance schedules are conducted as needed.
h. Assure no investigator's supplies or trash is left in the room.
i. Check supplies (e.g., soap, paper towels, etc.).
j. Clean counter/sink area and wipe down with disinfectant as needed.
k. Sweep floor as needed and empty trash.
l. Mop floor with AQUALIFE Multi-Purpose Cleaner dilution with water only.
m. Adult fish that are sick and need to be euthanized

E. Weekly Duties

a. Check $N0_2$
b. Check $N0_3$
c. Check NH_3
d. Spot clean tanks
e. Check water levels
f. Number of fish recorded
g. Check genotyping tanks and email lab if fish have been housed longer than one week.

F. Bi-weekly Duties
a. Check heating elements.
b. Check Drum Filter
c. Check UV lamp
d. Check Conductivity probe
e. Check pH probe

G. Tissue Collection for Genotyping

A. Fin clipping for DNA analysis and/or genotyping must be described in the ASP and approved by the IACUC

B. Individuals performing the procedure must be trained and show proficiency in the technique.

C. Zebrafish usually undergo tissue collection > 2month old. The caudal (tail) fin is the preferred tissue collection site. When done correctly, the fin will not bleed and should regenerate in 7-10 days.

D. The fin biopsy length should be limited to the smallest amount possible. In general, approximately 2-3mm of the caudal fin is sufficient to generate DNA for multiple PCR reactions and to prevent damage to the fin. For other fins, smaller amounts of tissue collected is recommended.

E. Anesthesia is frequently used to assist with fish handling.[12]

F. Other alternative methods for genotyping are available. [13, 14]

H. Euthanasia Guidelines

A. Euthanasia of animals is an important consideration in all ASPs and must be described. The PI and all investigators on the protocol must be trained in the proper technique, equipment, and agents for euthanasia and will be held responsible for the correct implementation of these guidelines.

B. All methods of euthanasia of zebrafish described below are the most commonly used methods in the Division of Intramural Research and are in accordance with the AVMA Guidelines on Euthanasia: 2020 Edition.[15] IUCAC may require scientific justification for other euthanasia methods.

C. The effectiveness of euthanasia methods may vary by life stage. Using adjunctive methods to guarantee death is recommended.

D. Zebrafish carcasses should be disposed of as Medical Pathological Waste according to NIH policies. (See Euthanasia Procedure)

I. Euthanasia Procedures

1. For zebrafish embryos/ larvae ≤ 7 dpf, euthanasia can be completed using one of the following methods:

A. Sodium Hypochlorite (bleach):

i. They should remain in this solution at least five minutes prior to disposal to ensure death. *Extreme caution must be used with this method to avoid any possibility of bleach entering the aquatic housing system water.

ii. For bleach solutions, verify the sodium hypochlorite concentrations:

1. 6.15% sodium hypochlorite, add to the culture system water at 1 part bleach to 5 parts system water.
2. 8.25% sodium hypochlorite, add to the culture system water at 1

part bleach to 7 parts system water.

B. Rapid chilling (hypothermic shock) followed by an adjunctive method of immersion in dilute sodium hypochlorite or calcium hypochlorite solution (2-step method).

2. For zebrafish larvae up to 8-15 dpf euthanasia requires a secondary method in order to ensure death. This age group 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.

A. Euthanasia by rapid chilling with adjunctive method:[16]

i. Submersion into ice water bath (5 parts ice/1 part water, 2-4°C) for at least 20 minutes to ensure death.

1. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice slurry as a barrier.

2. Since some zebrafish up to 15 dpf can survive rapid chilling even after prolonged absence of a heartbeat, a secondary method should be used to ensure death in embryos <15 dpf.

ii. Adjunctive methods after fish have been rendered unconscious prior to their application are freezing or other physical or chemical methods for destroying the brain function.

B. General anesthesia with pharmaceutical grade buffered MS-222 followed by adjunctive methods such as freezing or other physical or chemical methods for destroying the brain function.

3. For zebrafish 15 dpf, euthanasia can be done using the following methods:

A. Euthanasia by rapid chilling [17,18]:

a. Submersion in ice water bath (5 parts ice/1part water, 2-4°C) for at least ten minutes after cessation of opercular (i.e., gill) movement.

i. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice-slurry as a barrier.

ii. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 30 minutes after cessation of
all movement to ensure death by hypoxia.

b. Water temperature must be monitored with a thermometer and maintained between 1-4° C to ensure proper euthanasia

B. Overdose by prolonged immersion in buffered pharmaceutical-grade MS-222 in 250-500 mg/L solution.

a. Buffering with sodium bicarbonate should result in a pH between 7.0 and 7.5. Non-buffered MS-222 is acidic and causes an aversive reaction in unanesthetized fish.

b. Fish should be left in the solution for at least 30 minutes following cessation of opercular movement.

c. Fish should not be left in the euthanasia solution unattended, nor should they be left in the solution for more than one hour.

C. Anesthesia with pharmaceutical grade buffered MS-222 (100-200 mg/L) followed by an adjunctive method after fish have been rendered unconscious

prior to their application are decapitation, exsanguination, freezing or other physical or chemical methods for destroying the brain function.

APPROVED: **DATE:**

References:

1. OLAW. *Public Health Service Policy on Humane Care and Use of Laboratory Animals*.
(2015).
2. OLAW. *Frequently Asked Questions* https://olaw.nih.gov/guidance/faqs, 2017).
3. Dirian, L. *et al.* Spatial regionalization and heterochrony in the formation of adult pallial
neural stem cells. *Dev Cell* **30**, 123-136, doi:10.1016/j.devcel.2014.05.012 (2014).
4. Mione, M., Baldessari, D., Deflorian, G., Nappo, G. & Santoriello, C. How neuronal migration contributes to the morphogenesis of the CNS: insights from the zebrafish. *Dev*

Neurosci **30**, 65-81, doi:10.1159/000109853 (2008).
5. Furlan, G. *et al.* Life-Long Neurogenic Activity of Individual Neural Stem Cells and Continuous Growth Establish an Outside-In Architecture in the Teleost Pallium. *Curr Biol*
27, 3288-3301.e3283, doi:10.1016/j.cub.2017.09.052 (2017).
6. Valente, A., Huang, K.-H., Portugues, R. & Engert, F. Ontogeny of classical and operant
learning behaviors in zebrafish. *Learn Mem* **19**, 170-177, doi:10.1101/lm.025668.112 (2012).
7. Bretaud, S. *et al.* A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. *Neuroscience*
146, 1109-1116, doi:10.1016/j.neuroscience.2006.12.073 (2007).
8. Dreosti, E., Lopes, G., Kampff, A. R. & Wilson, S. W. Development of social behavior in
young zebrafish. *Front Neural Circuits* **9**, 39-39, doi:10.3389/fncir.2015.00039 (2015).
9. Perathoner, S., Cordero-Maldonado, M. L. & Crawford, A. D. Potential of zebrafish as a
model for exploring the role of the amygdala in emotional memory and motivational behavior. *J Neurosci Res* **94**, 445-462, doi:10.1002/jnr.23712 (2016).
10. Kossack, M. E. & Draper, B. W. Genetic regulation of sex determination and maintenance
in zebrafish (Danio rerio). *Curr Top Dev Biol* **134**, 119-149, doi:10.1016/bs.ctdb.2019.02.004 (2019).
11. Siegfried, K. R. & Draper, B. W. in *The Zebrafish in Biomedical Research* (eds Samuel C.
Cartner *et al.*) 151-164 (Academic Press, 2020).
12. De Lombaert, M. C., Rick, E. L., Krugner-Higby, L. A. & Wolman, M. A. Behavioral Characteristics of Adult Zebrafish (Danio rerio) after MS222 Anesthesia for Fin Excision. *J*
Am Assoc Lab Anim Sci **56**, 377-381 (2017).
13. Breacker, C., Barber, I., Norton, W. H., McDearmid, J. R. & Tilley, C. A. A Low-Cost Method
of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. *Zebrafish*
14, 35-41, doi:10.1089/zeb.2016.1348 (2017).
14. Wilkinson, R. N., Elworthy, S., Ingham, P. W. & Eeden, F. J. M. v. A method for highthroughput
PCR-based genotyping of larval zebrafish tail biopsies. *BioTechniques* **55**, 314-316, doi:10.2144/000114116 (2013).
15. Panel on Euthanasia. AVMA Guidelines on Euthanasia: 2020 Edition (2020).
16. Strykowski, J. L. & Schech, J. M. Effectiveness of recommended euthanasia methods in
larval zebrafish (Danio rerio). *J Am Assoc Lab Anim Sci* **54**, 81-84 (2015).
17. Matthews, M. & Varga, Z. M. Anesthesia and euthanasia in zebrafish. *ILAR journal* **53**, 192-
204, doi:10.1093/ilar.53.2.192 (2012).
18. Wilson, J. M., Bunte, R. M. & Carty, A. J. Evaluation of rapid cooling and tricaine

methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). *J Am Assoc Lab Anim Sci* **48**, 785-789 (2009).