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Effectiveness of Recommended Euthanasia Methods in Larval Zebrafish (*Danio rerio*)

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The popularity of zebrafish and its use as a model organism in biomedical research including genetics, development, and toxicology, has increased over the past 20 y and continues to grow. However, guidelines for euthanasia remain vague, and the responsibility of creating appropriate euthanasia protocols essentially falls on individual facilities. To reduce variation in experimental results among labs, a standard method of euthanasia for zebrafish would be useful. Although various euthanasia methods have been compared, few studies focus on the effectiveness of euthanasia methods for larval zebrafish. In this study, we exposed larval zebrafish to each of 3 euthanasia agents (MS222, eugenol, and hypothermic shock) and assessed the recovery rate. Hypothermic shock appeared to be the most effective method for euthanizing zebrafish at 14 d after fertilization; however, this method may not be considered an efficient method for large numbers of larval zebrafish. Exposure to chemicals, such as MS222 and eugenol, were ineffective methods for euthanasia at this stage of development. When these agents are used, secondary measures should be taken to ensure death. Choosing a euthanasia method that is effective, efficient, and humane can be challenging. Determining a method of euthanasia that is suitable for fish of all stages will bring the zebrafish community closer to meeting this challenge.

Abbreviations: dpf, days postfertilization; MS222, tricaine methanesulfonate.

Despite the increasing popularity of zebrafish (*Danio rerio*) as a model species for human diseases, guidelines for euthanasia remain vague, and the responsibility of creating appropriate euthanasia protocols essentially falls on individual facilities. As a result, euthanasia methods may vary between facilities and could lead to experimental variance. Although various euthanasia methods have been compared, few studies focus on the effectiveness of euthanasia methods regarding larval zebrafish. For example, one study evaluated the effectiveness of hypothermic shock and tricaine methanesulfonate (MS222) as agents of euthanasia, but only adult fish of various ages and a single concentration of MS222 were used.[11] MS222 is a chemical commonly used for anesthesia and euthanasia in zebrafish because it results in cessation of muscle movements by preventing action potentials.[3]

It is important to consider the effects of euthanasia on young fish, given that the dominant tissue of oxygen uptake gradually changes from the skin to the gills during the first 3 wk of development.[8] As the gills become the primary location for the intake of oxygen, the surface area for oxygen absorbance decreases. As a result, the effectiveness of MS222 increased with age among larval zebrafish.[9] The standard solution for euthanizing zebrafish contains 300 mg/L MS222 buffered to a pH of 7.0.[5] It is recommended that adult fish remain in a solution of this concentration for at least 10 min after the cessation of opercular movement.[2] However, our facility noted that the hearts of larval zebrafish continued to beat even after 30 min or more in MS222. Due to variation in the effectiveness of MS222 to induce euthanasia in different species, the *AVMA Guidelines for the Euthanasia of Animals* recommends the use of secondary measures to ensure death.[1] For practical purposes, it would be best to determine the most effective dose and method of euthanasia for zebrafish of all stages to ensure a humane death without the need for secondary measures.

Other methods of euthanasia currently approved for use in zebrafish include hypothermic shock, which is induced by placing fish in an ice bath, and eugenol treatment, in which fish are submersed in a solution containing eugenol.

Hypothermic shock is thought to work by slowing the rate of cellular activities and neural impulses. In many exothermic species, rapid chilling is used to immobilize animals. For example, hypothermic anesthesia is an acceptable method for many procedures in neonatal mice.[1] Hypothermic shock was found to be a more effective method of euthanasia than was MS222 treatment, and fish exhibited fewer signs of distress.[11] However, these findings are relevant to adult zebrafish only. To date, no studies on hypothermic shock and zebrafish fry have been published. To fully understand the effectiveness of hypothermic shock for euthanasia, more comprehensive studies need to be completed. The *AVMA Guidelines for the Euthanasia of Animals* recommends maintaining fry (age, 4 to 7 d postfertilization [dpf]) in ice-chilled water at least 20 min after the loss of opercular movement.[1]

Eugenol is recommended as an anesthetic for zebrafish because it induces anesthesia quickly, and recovery rates may be higher than those after MS222 anesthesia.[4] The anesthetic properties of eugenol are due to its ability to modulate various ion channels in neural cells that are responsible for nociception, the generation of neuronal spikes, and synaptic transmission.[6] In addition, eugenol poses advantages when it comes to cost.[1] Eugenol is an acceptable method of euthanasia in fin fish, according to the *AVMA Guidelines for the Euthanasia of Animals*.[1] These guidelines recommend that fish remain in a eugenol solution for at least 10 min after the cessation of opercular movements.[1] However, some components of eugenol (that is, isoeugenol) have been labeled as potential carcinogens by the US Food and Drug Administration.[10] In addition, the use of eugenol as an effective method for euthanasia remains unclear.

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The Guidelines for Use of Zebrafish in NIH Intramural Research Programs suggests that death among zebrafish can be confirmed by the cessation of opercular movements.[2] However, this event can be difficult to discern in nonadult fish. In the current study, we instead used visualization of the heartbeat as a more direct marker of anesthetic depth and as an indicator of approaching tissue death. Loss of heartbeat occurs at a greater anesthetic depth than does loss of opercular movement.[1] In larval and adult zebrafish, tissue death due to lack of cellular oxygen causes irreversible death. An additional challenge in confirming death when euthanizing zebrafish fry at 14 dpf or younger is that their very small size can allow sufficient diffusion of oxygen into vital tissues to maintain viability even after prolonged (that is, 20 min) cessation of the heartbeat.

The NIH guidelines[2] also state that zebrafish fry develop advanced cognitive abilities during the second week of life. Therefore, zebrafish may perceive pain and discomfort even at an early age. To err on the side of caution, it should be assumed that zebrafish fry possess sufficient cognitive ability by 2 wk to experience discomfort during euthanasia processes. *The AVMA Guidelines for the Euthanasia of Animals* states that, "techniques should result in rapid loss of consciousness followed by cardiac or respiratory arrest and the ultimate loss of brain function."[1] To adhere to these guidelines, it is important to establish protocols that ensure a quick death and prevent recovery. It is also important to establish consistency among euthanasia methods to eliminate variation in experimental results after euthanasia. For zebrafish specifically, the *AVMA Guidelines*[1] recommends bleach solutions for embryos and fry at 7 dpf or younger. With regard to hypothermic shock, adults should be exposed to the ice bath for at least 10 min after cessation of opercular movements and fry 4 to 7 dpf at least 20 min after cessation.[1] Secondary measures should follow the use of hypothermic shock and MS222 in fry younger than 3 dpf.[1]

The goal of the current study is to determine the effectiveness and efficiency of 3 methods of euthanasia in 14 dpf zebrafish fry. In this context, effectiveness is the ability to provide irreversible death 100% of the time. Efficiency is considered to be the time and manpower needed to perform the task of euthanizing a large number (for example, 50 or more) of 14-dpf fry. Although compromising humane euthanasia for the sake of saving time is not an acceptable practice, a method that is quicker and easier to perform likely will be performed correctly more consistently when large numbers of animals are involved. In research settings using zebrafish or rodents, where large numbers of animals may be euthanized daily, the efficiency of a method is an important consideration in preventing noncompliance incidents, such as incomplete euthanasia.

For the current study, we hypothesized that hypothermic shock is the most effective method for euthanizing 14-dpf zebrafish fry. We believe that choosing this method will enable zebrafish users to adhere to the *AVMA Guidelines for the Euthanasia of Animals*.

Materials and Methods

Humane care and use of animals. All fish used in this study were obtained from protocols approved by the NIH Animal Care and Use Committee. All fish were wildtype (EK strain) and were approved for genetic studies under protocols established at the NIH; all fish used were genetically normal. Pre- and post-filtration sentinel fish are examined semiannually. *Pseudoloma neurophilia* is present in the system, but fish were asymptomatic at the time of the study. An animal study protocol was created specifically for fish used in this euthanasia study.

Housing and husbandry. Adult fish approximately 6 mo old were housed in 1.8-L tanks at a density of no more than 18 fish per tank. These adult fish were used to produce the fry used in this study. Tanks were maintained on a recirculating system (Aquaneering, San Diego, CA). System water was obtained from municipal facilities and pretreated via carbon and resinous filtration followed by reverse-osmosis filtration. Recirculating system water was treated with a pressurized bead mechanical filter, a fluidized bed biofilter, and 4 ft. × 6 in. 25-µm polishing filters and exposed to 200 mJ/cm^2 UV lights (Emperor Aquatics, Pottstown, PA). Room lights were maintained on a 14:10-h light:dark cycle. Room and water temperatures were maintained at 82 °F ± 3 °F (27.8 ± 1.2 °C). Conductivity of the system water was maintained at 1000 µS, and pH was maintained at 7.3 ± 0.3. A 10% water change occurred via an automatic system every day. All parameters were monitored daily (YSI Water Quality Probes, Yellow Springs, OH), and pH was verified weekly (LaMotte, Chestertown, MD). In addition, ammonia, nitrite, nitrate, and alkalinity were monitored on a weekly basis by using a colorimeter (Hach, Loveland, CO). Adult fish were fed a commercial diet (Adult Zebrafish Diet, Ziegler Brothers, Gardners, PA) once daily and brine shrimp (*Artemia* spp.) twice daily. Brine shrimp eggs (San Francisco strain; Brine Shrimp Direct, Ogden, UT) were decapsulated on site.

Zebrafish fry were housed in 6-L tanks at a density of 120 fish per tank. The fry were placed on racks in the fish room at 5 dpf and fed rotifers once daily until 10 dpf. Water temperature was maintained solely by room temperature during this time. At 10 dpf, fry began acclimating to system water (with the parameters as described earlier) and were fed brine shrimp and a commercial diet (AP100, Zeigler) in the morning and brine shrimp and hatchfry (Argent Aquaculture, Redmond, WA) in the afternoon.

Study design. Four male and 4 female adult fish were bred in groups. These fish were from an EK strain that was developed in the Weinstein lab (Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, MD) from fish originally obtained from Ekkwill Farms (Ruskin, FL) in 1998. The strain has been maintained in the laboratory for more than 15 generations and has been selected for reproductive performance and lack of recessive lethal mutations. Embryos were collected and incubated in methylene-blue–treated water at 28.5 °C until 5 dpf. Methylene-blue–treated water was made by dissolving 0.001mg/L methylene blue (Sigma, St Louis, MO) and 0.06 g/L salt (Instant Ocean, Blacksburg, VA) in reverse-osmosis–treated water. At 5 dpf, fry were placed on racks in the fish room described earlier.

Fish were euthanized at 14 dpf according to 3 different acceptable methods.[1] All guidelines for euthanasia were followed, and carcasses were frozen, placed in a box for medical pathologic waste, and incinerated for disposal, according to facility procedures. The 3 methods of euthanasia used in this study were MS222 (tricaine), eugenol (clove oil), and hypothermic shock; 10 fish were exposed to each treatment. In our zebrafish facility, the lethal dose (300 mg/L) of MS222 is 5 times that of the dose used for anesthesia; we therefore exposed fish to 3 concentrations of MS222 (Western Chemical, Ferndale, WA) buffered with sodium bicarbonate to a final pH of 7.0: 300, 600, and 900 mg/L. Eugenol (Aqui-S 20E, 10% eugenol) was obtained from Aquatactics Fish Health (Kirkland, WA). The lowest dose of eugenol used was 5 times that of the dose used for anesthesia according to the manufacturer's guidelines. A total of 3 different concentrations of eugenol (pH 7.0) were used: 500, 1000, and 1500 µL/L. For fish euthanized by hypothermic shock in ice water (5 parts ice to 1 part system water), ice was obtained from

a laboratory ice maker supplied by municipal water that passes through a particulate and carbon filter, rendering it chlorine-free. The temperature of the ice bath was monitored by using a thermometer and was maintained at 0 °C.

For each treatment, fish were placed in a culture dish and observed collectively for at least 15 s every 5 min under a dissecting microscope. The time (in minutes) needed for the heart to stop beating was recorded. Experiments were terminated after 75 min when a heartbeat was still detectable. Fish remained in each euthanasia solution for 20 min after the point at which the heart stopped beating; they then were returned to system water and observed for 30 min.

Statistics. Time until cessation of the heartbeat was compared between euthanasia methods by using a one-way ANOVA followed by the Tukey multiple-comparison test (Prism 5.0, GraphPad Corporation, San Diego, CA). The assumptions for normality were met for this dataset. The Statistical Analysis System (SAS Institute, Cary, NC) was used to perform Fisher exact tests to discern significant differences in recovery rates between the 3 methods of euthanasia. A P value of 0.05 or less was considered to indicate a significant difference.

Results

Survival rate after MS222 treatment. To assess the efficacy of MS222 for euthanizing zebrafish, 10 zebrafish fry (14 dpf) were placed in a euthanasia bath containing 300 mg/L MS222. After 75 min, beating hearts were still detectable in all 10 fish, indicating that this dose of MS222 is not effective for euthanasia of zebrafish at this stage. Similar results were obtained when the tricaine dosage was increased to 600 mg/L. At 900 mg/L, the hearts of all 10 fish stopped beating within 10 min of entering the solution (Figure 1). However, when returned to system water, the heart beats of all 10 fish had returned after 30 min, suggesting that even at the highest dose tested, MS222 is not an effective means of euthanizing zebrafish fry (Figure 2).

Survival rate after eugenol treatment. Next, we examined the efficacy of eugenol as a euthanizing agent by placing zebrafish fry in a euthanasia bath containing 500 µL/L eugenol. After 75 min, all fish survived as indicated by the observation of beating hearts. Increasing the eugenol concentration to 1000 µL/L did not decrease survival. When 1500 µL/L eugenol was used, the hearts of all 10 fish stopped beating between 45 and 50 min after fish were placed in the solution (Figure 1). When returned to fresh system water, the heartbeats of only 3 fish returned (Figure 2).

Survival rate after hypothermic shock. To ascertain the effectiveness of hypothermic shock for euthanizing larval zebrafish, fry were placed in an ice bath containing 5 parts ice to 1 part system water. The hearts of all 10 fish stopped beating within 40 min of immersion in the ice bath (Figure 1). No heartbeats were observed in any fish after being returned to normal system water (Figure 2), demonstrating that hypothermic shock is an effective means of euthanasia for 14-dpf zebrafish.

Statistical analysis. In terms of efficiency, MS222 at 900 mg/L arrested the heartbeat most rapidly compared with 1500 µL/L eugenol ($P < 0.05$) and hypothermic shock ($P < 0.05$). However, MS222 was the least effective method for euthanizing 14-dpf fry when compared with eugenol ($P < 0.05$) and hypothermic shock ($P < 0.05$). Hypothermic shock significantly ($P < 0.05$) reduced the time until the cessation of the heartbeat, compared with eugenol. In addition, hypothermic shock was the most effective method for euthanizing 14-dpf fry because none of these fry regained a heartbeat.

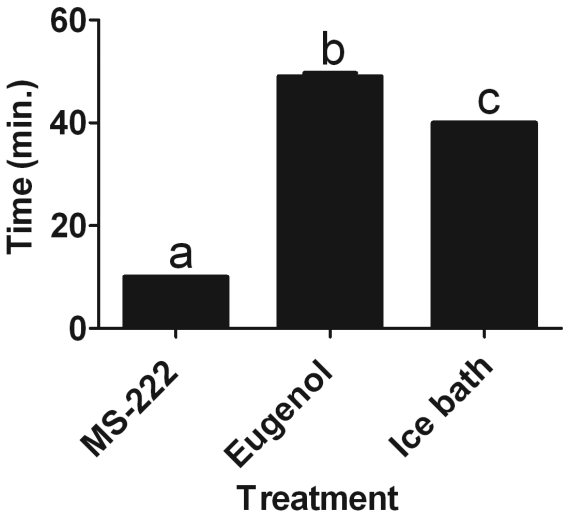

Figure 1. Time (min; mean ± SEM [$n = 10$]) until cessation of the heartbeat in 14-dpf zebrafish fry after exposure to MS222 (900 mg/L), eugenol (1500 µL/L), and hypothermic shock (ice bath). Different letters indicate significant ($P < 0.05$) differences in time until cessation of the heartbeat.

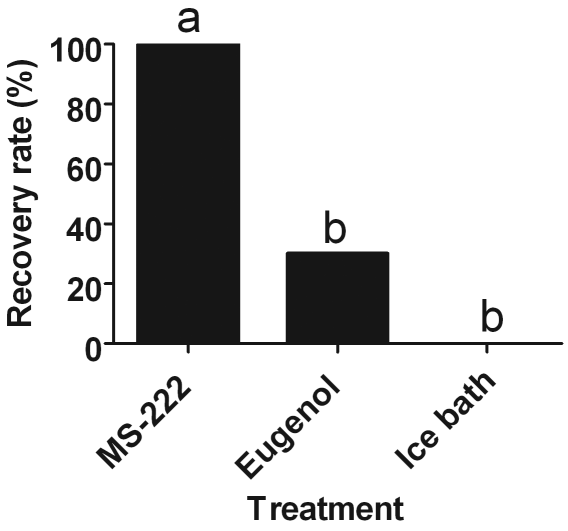

Figure 2. Recovery rate (%; $n = 10$) of 14-dpf zebrafish fry after exposure to MS222 (900 mg/L), eugenol (1500 µL/L), and hypothermic shock (ice bath). Different letters indicate significant ($P < 0.05$) differences in recovery rate.

Discussion

This study investigated 3 methods of euthanasia among zebrafish fry (MS222, eugenol, and hypothermic shock). Compared with adults, zebrafish fry are typically less susceptible to death by chemicals such as MS222.[9] Our facility noted prolonged survival of 14-dpf zebrafish in a standard euthanasia bath of 300 mg/L MS222. In the current study, the hearts of 14-dpf zebrafish continued to beat for more than 1 h after immersion in MS222 at concentrations of 300 and 600 mg/L. Only the highest dose of 900 mg/L effectively resulted in cardiac arrest within 10 min. However, MS222-treated 14-dpf fish were able to recover their heartbeat after immersion in a recovery tank with clean system water. Because fry exposed to MS222 revived when returned to untreated system water at normal housing temperature, a secondary method of euthanasia should be used while fry are still anesthetized. For adult fish, maceration is an acceptable method; however, given the small size of fry, the equipment and

methods typically used on adults might not be 100% effective for fry.[1] One practical method may be the denaturation of fry, accomplished by adding bleach (dilution, 1:5) directly to the euthanasia solution containing the anesthetized fry.

Because of the chemical's recent acceptance for use in fish euthanasia and success as an anesthetic, we assessed the effectiveness of eugenol to induce euthanasia in larval zebrafish.[1,4] Similar to MS222, only the highest concentration of eugenol tested (1500 µL/L) effectively induced cardiac arrest in 14-dpf zebrafish. Even though it was only 70% effective overall, 1500 µL/L eugenol was more effective than was 900 mg/L MS222 for euthanizing zebrafish fry. Although fewer fish recovered after immersion in this eugenol solution, the time to reach cardiac arrest was significantly longer ($P < 0.05$) than that of zebrafish fry exposed to 900 mg/L MS222. In addition, various components of eugenol (for example, isoeugenol) have also been labeled as potential carcinogens by the US Food and Drug Administration.[10] Given the prolonged duration fish would have to remain in a highly concentrated eugenol solution and that eugenol was only 70% effective in euthanizing 14-dpf fry, this method likely will not be suitable for many facilities. In addition, the use of eugenol must be accompanied by secondary measures to ensure appropriate euthanasia.

Recent publications have indicated that adult zebrafish show aversion to MS222 and eugenol-related compounds.[7,12] It is beyond the scope of the current study to evaluate the aversion of zebrafish fry to commonly used anesthetics. Additional studies are needed to evaluate whether fry 14 dpf and younger display the same aversion as do adult zebrafish. These future studies should take into consideration that aversion is not equivalent to pain and distress.[1] It is normal behavior in most animals to avoid a distinctly unfamiliar change in their environment. Rodents, nonhuman primates, and other mammals exposed to inhalant anesthetics such as isoflurane show aversive behavior, yet isoflurane is considered a humane and effective anesthetic in these species and not a painful stimulus.[1]

Hypothermic shock was the only form of euthanasia in the current study that prevented larval zebrafish from recovering after cardiac arrest. However, the time needed to achieve cardiac arrest via hypothermic shock was comparable to that of fish immersed in 1500 µL/L eugenol and significantly ($P < 0.05$) longer than that of fish immersed in 900 mg/L MS222. Therefore, hypothermic shock was not the most efficient method for euthanasia of zebrafish fry, but it was 100% effective. Other studies suggest that hypothermic shock may result in fewer stress indicators in adult zebrafish.[11] Although our current study did not focus on stress indicators, the potential for hypothermic shock to become standard practice in euthanizing zebrafish fry, as well as other life stages, is well supported. This method would not require secondary measures to ensure appropriate euthanasia, especially when fish are maintained in the ice bath for at least 40 min. In addition, ice buckets are easily maintained when monitored correctly. When buckets are well-insulated, we have found that ice rarely has to be added throughout an 8-h period.

In the context of biologic research performed on large-scale study populations, numerous zebrafish may need to be euthanized, and the time to perform such tasks is often limited. It is imperative to determine the most effective and efficient method for euthanasia among all life stages of zebrafish to increase efficiency in large zebrafish facilities that already require intense husbandry by a large team. For example, we know anecdotally that many zebrafish facilities store fish frozen and then dispose of them according to facility requirements, thus further decreasing the potential for recovery. It is also imperative that differences in euthanasia methods not confound experimental results from various zebrafish facilities.

Future experiments with younger fish and adults will help determine a standard method of euthanasia for zebrafish users. As the use of zebrafish in biologic research continues to expand, a standard method of euthanasia will benefit all zebrafish users and increase the validity of experimental results from studies that use zebrafish as a model. According to the results of this study, hypothermic shock appears to be the most effective method of euthanasia for zebrafish fry, and its use will enable zebrafish users to adhere more closely to the *AVMA Guidelines for the Euthanasia of Animals*.[1]

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