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Effect of 3 Euthanasia Methods on Serum Yield and Serum Cortisol Concentration in Zebrafish (*Danio rerio*)

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Zebrafish are an important model in neuroscience and developmental biology and are also an emerging model in hematology and immunology. Little information is available for zebrafish regarding the physiologic impact of different euthanasia methods and whether a chosen method of euthanasia can impact serum yield. These parameters could impact the choice of euthanasia method for a study. To that end, the current study compared 3 methods of adult zebrafish euthanasia and their effects on 3 distinct criteria; time to loss of opercular movement, volume of serum obtained, and serum cortisol concentration. Blood was collected using a postmortem tail amputation and centrifugation blood collection technique. Time to loss of opercular movement differed significantly among euthanasia methods, with animals undergoing rapid chilling displaying the shortest time (mean Rapid Chilling: 40 s; Benzocaine: 86 s; MS222: 96 s). All methods of euthanasia resulted in a comparable average serum yield (Rapid Chilling = 7.5 µL; Benzocaine = 8.5 µL; MS222 = 7.5 µL per fish). None of the euthanasia methods tested resulted in average cortisol concentrations above the reported physiologic range. Although no significant differences were observed in serum yield or serum cortisol concentration, rapid chilling remains the preferred method for painless, humane euthanasia.

Abbreviations: MS222, tricaine methanesulfonate; SSRI, selective serotonin reuptake inhibitor

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Zebrafish are a common model in biology and neuroscience research, particularly in studies of development, toxicology, and carcinogenesis.[19,40] Their small size, external fertilization, and transparent embryos make them ideal subjects to visualize early developmental changes.[19,39] In addition, the availability of a fully sequenced genome makes zebrafish attractive models for multiple other fields, such as immunology and hematology.[8,19,23] Acute stress-related changes to blood chemistry related to euthanasia may present a confounding variable in neurophysiology, immunology or other fields. In many such studies, blood or serum is collected for cytologic and biochemical testing, such as protein or enzyme assays, DNA sequencing or RNA expression profiles.[4,16,24] Due to their small size, recovering serum from zebrafish in the volume required to perform serum-based assays is challenging. In some cases, assays may require more volume than can be obtained from a single zebrafish. A recent study reported a novel, postmortem centrifugation technique to recover blood from individual fish and subsequently separate out the serum.[4,12] However, this refinement yielded different volumes of blood after zebrafish euthanasia with either MS222 or clove oil, a eugenol-based anesthetic.[12] In this same manuscript, a significant increase in serum recovery was reported when using clove oil as a euthanasia agent when compared with MS222, which was posited to be due to intrinsic anticoagulant properties of eugenol.[12] Comparisons of postmortem blood or serum collection efficiency after other euthanasia methods have not been published.

The AVMA Guidelines for the Euthanasia of Animals: 2013 Edition details a number of acceptable methods for the euthanasia of zebrafish, including MS222 (tricaine methanesulfonate), benzocaine solution, and rapid chilling in 2 to 4 °C water.[3] MS222 and benzocaine are both local anesthetics that inhibit motor and sensory nervous responses by blocking sodium uptake and membrane excitability in neurons.[9,10,22,27] Anesthetic overdose causes loss of nervous input required for opercular movement and respiration.[3,10,27] Similarly, rapid chilling also causes a slowing of nerve conduction, resulting in loss of righting reflex and opercular movement in tropical poikilotherms, such as zebrafish.[20,40] Prior evaluation of euthanasia methods in zebrafish have focused on time to loss of righting reflex, time to loss of opercular movement and behavioral aversion measures to gauge their effects on the welfare of fish.[40,41] Although the efficacy of euthanasia has been examined in zebrafish, few publications included a physiologic comparison of samples generated by these different methods of euthanasia.[10,12,16,29,40]

The hormone cortisol is a widely accepted indicator of physiologic stress in fish, as well as other vertebrate species.[28,29] Whole-body cortisol has also been validated as a marker of acute and chronic stress in zebrafish, related to crowding, transport, air exposure, and netting.[28,29,35] In addition, selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines have been shown to inhibit stress induced increase in serum cortisol a dose dependent manner.[1,13] These responses are consistent with those seen in mammalian species, including humans. Interestingly, a human salivary cortisol ELISA has been used in recent publications to detect cortisol in zebrafish.[12,16] This kit is inexpensive,

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readily available commercially, and easy to use, but ranges for serum cortisol concentrations of zebrafish under different conditions are still being explored using this method.[12,16] In some studies, eugenol-based anesthetics have been shown to depress cortisol production and stress-related glucose responses, but not in others, mostly in comparison to MS222.[12,18,41] Comparisons of MS222 with other methods of euthanasia are required to further characterize common alternatives. In a study comparing euthanasia by rapid chilling to overdose of MS222, rapid chilling showed shorter latency to loss of movement, minimal signs of distress, and no obvious tissue damage on histopathology, but cortisol was not measured.[40] Measuring cortisol after euthanasia with these common methods provides useful information for future research where cortisol may confound study data.

The aim of our study is to compare 3 different methods of euthanasia: MS222, rapid chilling, and benzocaine and the relative effect of these techniques on 3 criteria: time to loss of opercular movement, serum collection efficiency, and serum cortisol concentration. In addition to comparing the serum cortisol level after each euthanasia method, we sought to further support the use of a tail amputation and centrifugation technique for blood or serum collection and a human salivary cortisol ELISA to detect cortisol concentration, both of which represent refinements to traditional methods.[4,12,16] We hypothesized that rapid chilling would have a significantly shorter time to loss of opercular movement than the other methods, that all methods would yield similar serum volumes using refined blood collection technique, and that all euthanasia methods would yield serum cortisol concentrations within the published physiologic range. None of the methods used had the presumed anticoagulant effect previously observed with clove oil derivatives, so we did not expect a significant difference between the chosen methods with respect to serum collection. Because all methods cause loss of opercular movement within a short period of time (less than 5 min), we did not expect they would cause an increase in cortisol levels beyond the physiologic range.

Materials and Methods

Humane Care and Use of Animals. Animals were housed in an AAALAC-accredited animal facility in compliance with the *Guide for the Care and Use Of Laboratory Animals*.[17] All animals were euthanized in accordance with AVMA guidelines, under a protocol approved by New York University Langone Health's Institutional Animal Care and Use Committee. Blood sampling was performed postmortem.

Animals and Housing. Adult (older than 3 mo) male and female zebrafish (*Danio rerio*) of mixed genotypes were used, including AB, TL, TU, EK, and WIK backgrounds and incrosses. The fish were culled from available breeding colonies. To our knowledge there was no relevant difference between the strains. All fish were apparently health, bred inhouse and were experimentally naïve. Fish were originally obtained from a variety of academic and vendor sources. Fish not obtained from SPF vendor sources were quarantined and embryos bleached before transferring to the main recirculating system. The experimental design did not control for genetic background or sex specifically. Fish were reared according to published guidelines.[17,39] Animals were housed in mixed sex groups of 30 to 40 fish in 12-L tanks on a recirculating system at 27 ± 1 °C. The room in which fish were housed was maintained on a 14:10 light:dark cycle. System water was treated by reverse osmosis (RO) with added salt (Instant Ocean, Blacksburg, VA) and sodium bicarbonate (Sigma Aldrich, St Louis, MO) buffer. System water was passed through mechanical, biologic, chemical and UV filtration. Water was kept within the following parameters: pH 6.8 to 7.9, conductivity 400 to 800 µS/cm, total gas pressure (TGP) 95 to 99, ammonia at 0 ppm, nitrate at 0 ppm and nitrate at less than 5. Adult fish were fed twice daily using artemia (Brine Shrimp Direct, Ogden, UT) and a commercial flake diet (TetraMin Tropical Flakes, Tetra, Blacksburg, VA). Spirulina flake and Cyclop-eeze (Argent Aquaculture, Redmond, WA) was fed once daily.

Euthanasia of Experimental Groups. Experimental groups of 10 to 12 fish (MS222: 5 cohorts; Rapid Chilling: 5 cohorts; Benzocaine: 7 cohorts) were randomly chosen from tanks located on the main recirculating system and were transferred to a 1-L holding container filled with recirculating system water. All fish were netted and euthanized during the same 2-h block (0930 to 1130) the morning of euthanasia, to account for cyclical changes in cortisol levels. Each experimental group was provided a minimum of 30 min of postnetting acclimation after placement in the 1-L holding container. Cohorts were then euthanized. Time to loss of opercular movement for all methods was measured by recording the observed time from immersion to cessation of opercular movement of the last fish in the cohort. Following euthanasia, 8 fish from each euthanasia group were selected for blood collection, based on visual similarity in size (between and among groups). Fish were removed from the tank within 1 min of loss of opercular movement and placed in centrifugation tubes as described below for blood collection; exsanguination served as a secondary physical method of euthanasia to improve physiologic data accuracy.[11] No animals responded to tactile or surgical stimulation or recovered opercular movement prior to centrifugation. For all euthanasia methods, at least 5 cohorts were examined. For the benzocaine group, 2 cohorts were repeated due to issues with ELISA data acquisition.

Euthanasia using MS222. The MS222 solution (Syndel Laboratories, Ferndale, WA) was prepared at a concentration of 800 mg/L. The solution was buffered to a pH of 6.5 to 7.5 with sodium bicarbonate. The euthanasia process was initiated by slowly (1 to 3s) adding the 800 mg/L buffered MS222 solution to the 1-L holding container at a ratio of 1:1 to yield a desired MS222 concentration of 400 mg/L. Fish were observed until cessation of opercular movement was noted.

Euthanasia by Rapid Chilling. Recirculating system water and ice were combined in a clean rodent static cage bottom (Tecniplast, West Chester, PA). Ice was added first, and then system water was used to fill the cage to a sufficient depth to cover the zebrafish. The ratio of ice to water was roughly 5:1. Ice and water were both measured volumetrically for a total volume of roughly 5 L (by mass, this was approximately 3.2 kg of ice water). A zebrafish breeding tank slotted insert was placed and partially submerged within the rodent static cage bottom. The temperature of the ice water slurry was monitored until a temperature range of 2 to 4 °C was verified.[40] When the temperature of the ice bath was verified to be within the low end of the target range, fish were added to the breeding tank insert by directly pouring the fish in a volume of approximately 120 to 140 mL of room temperature (19 to 21 °C) system water from the 1-L holding container. Throughout the euthanasia process, the temperature of the ice bath was monitored to ensure that it remained within 2 to 4 °C. Fish were observed until cessation of opercular movement was noted. All fish were in contact with the ice water for greater than or equal to 30s.[37]

Euthanasia with Benzocaine. Benzocaine (Sigma-Aldrich, St Louis, MO), 500 mg/L solution was made by dissolving benzocaine in ethanol and then subsequently adding the benzocaine/

ethanol solution to deionized water.[2,3] The benzocaine solution was brought to room temperature and pH was determined to be 6.5 to 7.5, prior to euthanasia.[2,3] The euthanasia process was initiated by slowly adding the 500 mg/L benzocaine solution 1:1 to a known volume of system water containing the fish to yield a final concentration of 250 mg/L.

Serum Yield. Blood was collected postmortem using a previously published technique.[4] Briefly, the tail was transected just cranial to the caudal fin and the remainder of the fish was placed with the cut surface resting in the well of a fenestrated 0.6 mL microfuge tube. The 0.6 mL tubes were nested inside of 1.5 mL tubes and centrifuged at 2110 RPM for 5 min at room temperature (Eppendorf 5425, Hamburg, Germany). At the end of the centrifuge cycle, fish were removed, established blood clots were removed by transecting the cut end of the tail and the centrifugation process was repeated. After the second centrifugation cycle fish were discarded along with the 0.6 mL tubes. The 1.5 mL tubes were then centrifuged at 12,400 RPM for 15 min to separate serum from solids. Serum volume recovery was measured by weighing microcentrifuge tubes with pooled samples before and after removal of serum for ELISA. Volume was verified during pipette recovery.

Cortisol ELISA. ELISA was performed either within 2 h of obtaining serum samples, or serum was stored at -80 °C. Serum cortisol concentrations were determined using a cortisol ELISA kit (Salivary Cortisol Enzyme Immunoassay Kit, Salimetrics, Carlsbad, CA), according to manufacturer's instructions.[12,16] Serum samples were pooled by experimental cohort to obtain the 25 µL sample volume required ELISA analysis. Cortisol concentration data were obtained by performing nonlinear regression of the enzyme immunoassay absorbance readings against a standard absorbance curve obtained on a plate reader (EnSpire, PerkinElmer, Waltham, MA).

Statistics. Data were analyzed using Excel Stats Package (Microsoft, Seattle, WA). Statistical significance was set to a P value of less than 0.05. Serum volumes were pooled by cohort to provide adequate sample volume for ELISA, and divided to find the average individual fish contribution. The effect of euthanasia method on serum cortisol concentration, time to loss of opercular movement, and recovered serum volume was analyzed by single factor ANOVA (2 tailed) with a Tukey posthoc test. For all metrics, pooled samples were used. Shapiro–Wilk normality testing was employed for cortisol data using R statistical software (R Foundation for Statistical Computing, Vienna, Austria).[33]

Results

Time to loss of opercular movement. Cessation of opercular movement was observed in a significantly shorter period of time after rapid chilling (40 s) as compared with benzocaine (86 s) and MS222 (96 s; $P < 0.05$) (Figure 1). Time to loss of opercular movement did not differ significantly between MS222 and benzocaine ($P = 0.78$) on posthoc testing.

Serum yield. Mixed sex cohorts of zebrafish were used to test the volumes of serum that could be collected. There was no statistically significant effect of euthanasia method on amount of serum recovered per cohort, or on average per fish, and no statistically significant difference between euthanasia methods ($P > 0.05$) (Figure 2).

Serum cortisol. Mixed sex cohorts of zebrafish were used to test the effect of euthanasia method on serum cortisol concentration. Serum cortisol concentration did not differ significantly between euthanasia methods as determined by single factor ANOVA ($P > 0.05$) (Figure 3). Posthoc testing did not reveal a significant difference between any pairs. Shapiro Wilk normality testing did not reject normality of data at 0.05 α level.

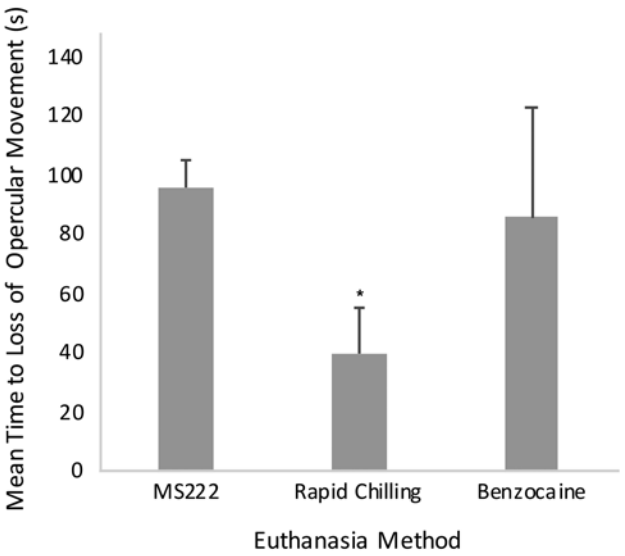

Figure 1. Analysis of the effect of euthanasia method on time to loss of opercular movement showed a significant effect ($P = 0.01$) by single factor ANOVA. Posthoc comparison of time to death between each form of euthanasia using, showed that rapid chilling had significantly different time to loss of opercular movement when compared with MS222 or benzocaine ($P < 0.05$). The significantly different value is marked with an (*). Using the same method, MS222 did not differ from benzocaine ($P > 0.05$). Error bars reflect the standard deviation of each mean. Statistical analysis was performed based on cohort as the n. There were 5 cohorts of rapid chilling and MS222 euthanasia and 7 for benzocaine (MS222 $n = 5$, rapid chilling $n = 5$, benzocaine $n = 7$).

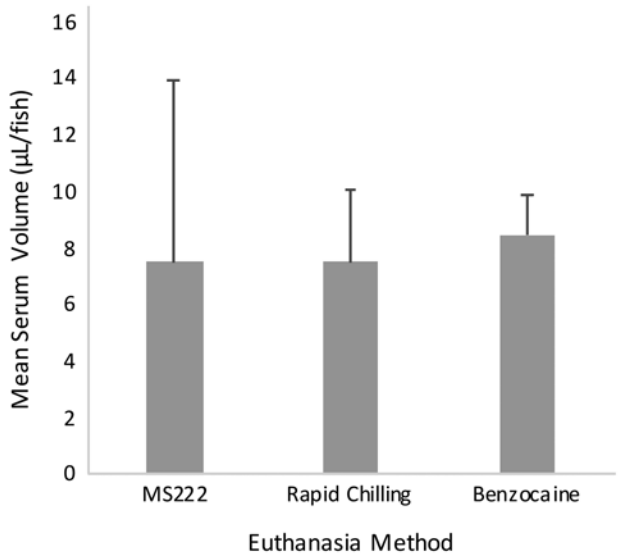

Figure 2. Single Factor ANOVA also showed no significant effect of euthanasia method on serum yield per fish ($P > 0.05$). Error bars reflect the standard deviation. Raw data were divided by the number of fish per measured volume to find a serum volume/fish value as the relevant metric of interest, contributing to a grand mean, and represented per fish (MS222 $n = 16$; rapid chilling $n = 16$; benzocaine $n = 40$ fish).

Discussion

MS222, rapid chilling and benzocaine are all acceptable forms of zebrafish euthanasia per the AVMA guidelines.[3] Based on previous publications, we hypothesized that rapid chilling

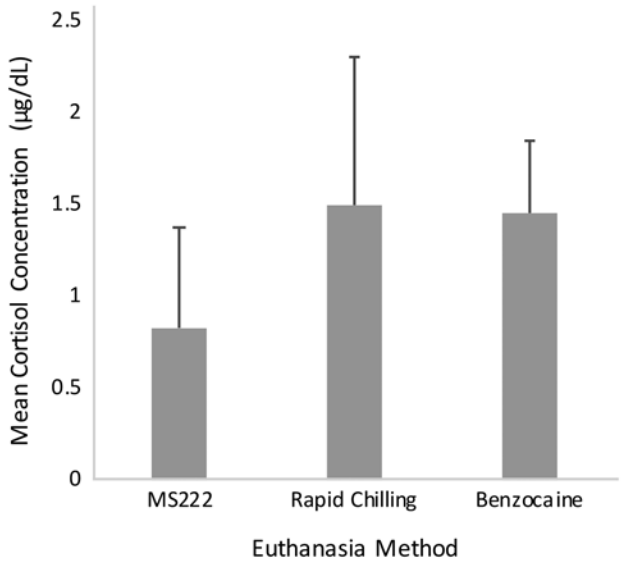

Figure 3. Between groups ANOVA showed no effect of euthanasia method on mean serum cortisol concentration or significant differences among methods ($P > 0.05$). Error bars reflect the standard deviation. Statistical analysis was performed by euthanasia method based on pooled, readable serum samples. Sample number per treatment varied due to differences in available serum volume and ELISA sample loss (MS222 $n = 15$; rapid chilling $n = 10$; benzocaine $n = 4$). Shapiro Wilk test for did not reject normality at a 0.05 α level.

would result in a shorter time to loss of opercular movement than MS222 or benzocaine.[22,37,40] We also hypothesized that shorter time to death would correlate with lower serum cortisol levels.[40] Rapid chilling resulted in the shortest average time to loss of opercular movement and was significantly faster than either MS222 or benzocaine. Our methods, which involved gross observation until the last fish in a cohort lost opercular movement, may have resulted in longer average times in comparison with previous studies. When comparing MS222 to benzocaine directly, the average time to loss of opercular movement was not significantly different between the 2 euthanasia methods. Prior to the latest edition of the AVMA Guidelines for the Euthanasia of Animals, rapid chilling was not recommended for unanesthetized zebrafish.[3] Studies assessing rapid chilling have shown shorter latency to loss of movement and fewer behavioral indications of stress than MS222.[40] European guidelines, however, do not condone this method and require special licensing to perform it in a laboratory setting.[36]

In our study, fish euthanized via rapid chilling were added to an ice water bath through the use of a pouring technique. The pouring technique used a small volume of room temperature water to gently transfer fish into a much larger volume of prepared ice water. Normally zebrafish are netted prior to euthanasia and are released directly into a prepared ice water bath. Because netting is a known acute stressor, we wanted to avoid netting stress as a potential confounder when comparing cortisol between different euthanasia methods.[29] When performing rapid chilling as a method of euthanasia, water temperature must be carefully monitored throughout the euthanasia process. Allowing water temperatures to rise above 4 °C during euthanasia can potentially have a detrimental effect on zebrafish welfare. As a precaution, our study first piloted the pouring technique without fish to ensure that the addition of small volumes of room temperature water would not adversely affect the overall temperature of the ice water bath used for rapid chilling. We would strongly recommend individual validation for any study seeking to replicate a similar pouring technique as a means of transferring fish for euthanasia. Many factors, such as exact mass of ice compared with water, or the surface area of ice, may affect the consistency of method at other institutions.

MS222 is widely used as an anesthetic and euthanasia agent for aquatic species in laboratory and aquarium settings. It is available in both pharmaceutical and nonpharmaceutical grade forms.[10,30] However, cost and difficulty obtaining the pharmaceutical grade version can make it less attractive to researchers compared with rapid chilling. MS222 is classified as a chemical hazard, particularly in its crystalline powdered form, and should be mixed in a hood with proper PPE to avoid irritation to eyes and mucous membranes.[10] Given the potential hazard of using MS222, our study suggests that rapid chilling may also be refinement of technique, just like the centrigugation technique to maximize blood volume.

Benzocaine hydrochloride is water soluble and commonly used by home aquarists for euthanasia of finfish. Benzocaine may be less preferentially used in research as a euthanasia agent because it is a nonpharmaceutical grade compound, and it requires dissolution in a nonaqueous solvent prior to use. During our study, fish in the benzocaine groups were observed to exhibit piping behavior and hypertaxia at a subjectively higher rate than the other 2 treatment groups, but the frequency was not quantified. Given the higher frequency of these abnormal, anxiety-like behaviors, and the high variability in latency to loss of opercular movement, benzocaine was the least desirable method of euthanasia among the those we tested from a welfare perspective.

In our study, we hypothesized that our selected euthanasia methods would show no significant differences in serum yield, in contrast to a study comparing MS222 and clove oil.[12] Confirming our hypothesis, all euthanasia methods provided comparable volumes of serum when using the amputation and centrifugation technique and were consistent with previous findings on blood and serum volume extracted per fish (4 to 11 µL).[4,12] Volume of serum obtained using the amputation and centrifugation technique does not appear to be influenced by the euthanasia methods we tested, but a comparison of serum yield after euthanasia between clove oil and rapid chilling or benzocaine would be of interest.[4,12]

Lastly, we hypothesized that all 3 euthanasia methods tested would display cortisol concentrations within the reported physiologic range because all methods have a short latency to death, possibly before the induction of the cortisol pathway, though that has not been confirmed for this species. This use of serum is a refinement on whole-body or gill homogenization for cortisol extraction.[5,15,29] Cortisol has been accurately and inexpensively measured using human salivary cortisol ELISA kits with both serum and whole body maceration methods.[12,13,16] Previous studies in other fish species have reported that lower, sedative doses of MS222 elicit similar cortisol responses to crowding stress, whereas higher doses that cause more rapid immobilization do not.[32,34] We hypothesized that doses appropriate for euthanasia would not induce an increase in cortisol. In previous studies, zebrafish euthanized with a eugenol-based agent (clove oil) show significantly lower baseline cortisol concentration, as compared with cohorts euthanized with MS222.[12] We did not observe a cortisol difference of this magnitude among the 3 methods of euthanasia. We did experience sample loss for benzocaine samples on this metric due to ELISA plate reader failure. However, the completed readings were tested for normality and included in a comparison between euthanasia methods. Our results are consistent with physiologic baseline cortisol

concentrations reported in previous studies, both from the same salivary immunoassay kit and other similar methods.[16] Our data provide further support for the use of the human salivary immunoassay kit for zebrafish serum cortisol detection.

Cortisol concentration at the time of death may not accurately reflect the stress level of fish in our study due to the dynamics of the cortisol response.[31] In zebrafish, the stress response is controlled by the HPI (hypothalamic-pituitary-interrenal) axis, which is a fish analog to the HPA (hypothalamic-pituitary-adrenal) axis in mammals).[6,7,31] In response to a stressful stimulus, zebrafish undergo a series of physiologic events resulting in cortisol secretion.[6,7,31] The acute cortisol response in fish is known to be fast in zebrafish (under 5 min) and to peak around 30 min.[14,26] However, since all fish lost righting reflex and opercular movement within 0.5 to 3 min, it is not clear whether cortisol would have had time to increase beyond physiologic levels. However, since we did not observe a demonstrable effect on cortisol after any of the selected methods of euthanasia, the euthanasia method alone should not induce a change in cortisol that could confound other physiologic data.

Assays for more acute biochemical markers of physiologic stress, such as catecholamine levels, or less saturable responses, such as direct ACTH or CRH levels, could further contribute to our understanding of the acute stress response in zebrafish overall and as it relates to accepted methods of euthanasia.[21] However, these responses often cannot be sampled repeatedly in the same individual zebrafish due to their size, unless they can be measured from the tank water. Catecholamine responses are often very fast, making sampling difficult, even in larger species.[7,25] When subjected to chronic stress, circulating ACTH and cortisol both decrease relative to their levels in acute stress conditions but remains above physiologic levels.[16,21,38] This is due to decreased intrarenal reactivity to ACTH and negative feedback of cortisol on the hypothalamus and pituitary.[21,38] Reported cortisol concentration ranges make us confident that our results do not represent HPI axis exhaustion.[6,16]

Based on the data from our study, we posit that any of the tested zebrafish euthanasia methods are appropriate with respect to volume of serum recovery and lack of interference from an acute cortisol response. Rapid chilling may be preferred over MS222 and benzocaine due to shorter latency to death, and obviation of chemical hazards. Our study also adds to the body of evidence supporting the tail amputation and centrifugation method for serum collection and the use of a human salivary cortisol ELISA for serum cortisol measurement in zebrafish.

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