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Evaluation of Effective and Practical Euthanasia Methods for Larval African Clawed Frogs (*Xenopus laevis*)

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Larval, or tadpole-stage *Xenopus laevis* frogs are a popular research model for developmental biology and disease studies. Existing euthanasia guidance documents offer recommendations for both eggs and adult stages, yet do not specifically address the larval stage. Data evaluating effective euthanasia methods for groups of *X. laevis* tadpoles would therefore be useful. The goal of the current study was to evaluate the efficacy of various immersion euthanasia procedures on tadpoles: tricaine methanesulfonate (MS222) at 6 g/L, eugenol at 800 µL/L and rapid chilling (2 to 4 °C). We also evaluated tadpoles at various developmental stages (NF stages 46, 47 and 49). Tadpoles (*n* = 70) were exposed to euthanasia solution for 15 min, and controls (*n* = 40) were placed in housing tank water for 15 min. All animals were then placed in recovery tanks containing housing tank water for 4 h to confirm irreversibility of each agent. Cessation of the heartbeat was assessed at the end of euthanasia solution exposure and at each hour thereafter. We found that immersion in a 6 g/L solution of MS222 resulted in 100% euthanasia of all larval stages tested. Conversely, eugenol produced variable euthanasia rates that were affected by both age group and batches of stock solutions. Rapid chilling was completely ineffective as a euthanasia method in our study. Based on our findings, we recommend MS222 as an effective and practical means of euthanizing large numbers of *X. laevis* tadpoles.

Abbreviations: MS222, tricaine methanesulfonate; NF, Nieuwkoop and Faber

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Various species of aquatic frogs, including *Xenopus laevis*, provide important models for biomedical research. The larval stages of these amphibians are commonly used in developmental biology and disease research.[10,18,26,30] Despite their use in research, published data regarding appropriate methods of euthanasia for larval forms of any amphibian species is scant. The *AVMA Guidelines for the Euthanasia of Animals* specifically recognizes the challenges in providing euthanasia methods for laboratory amphibians, and although specific recommendations are given for euthanizing adult amphibians and eggs, no guidance currently exists for larval (tadpole) forms.[2] Furthermore, to the authors' knowledge, no published studies have assessed euthanasia methods for larval *X. laevis* frogs. Therefore, a controlled study to provide recommendations for a practical, safe and efficacious way to euthanize *Xenopus* tadpoles would be valuable to those using these models.

The *AVMA Guidelines for the Euthanasia of Animals* and *The Guide for the Care and Use of Laboratory Animals* recommend that criteria that should be considered for euthanasia of animals are reliability, irreversibility, time until unconsciousness and death, and appropriateness for the species and age of the animal.[2,19] Another consideration is to use euthanasia methods that are more rapid and less complicated to complete, with the goal of reducing noncompliance in a research setting.[34] These considerations are paramount when developing a practical and robust euthanasia protocol for *X. laevis* tadpoles in a research setting.

Although the AVMA document does not provide guidance specific to euthanasia of amphibian larvae, the document does provide recommendations for the euthanasia of larval stages of various types of fish, including zebrafish (*Danio rerio*), and points out that different methods are more appropriate for different stages of larval development.[2] Although the physiology of zebrafish fry and *Xenopus* tadpoles differs, both species undergo changes in the physiologic mechanism of oxygen consumption as they develop. The effectiveness of tricaine methanesulfonate (MS222) appears to vary based on the gills replacing the skin as the primary site of oxygen intake in zebrafish larvae.[27] This raises the question of whether changes in respiratory physiology during development can affect euthanasia outcomes in *Xenopus* larvae.

The developmental stages of *X. laevis* tadpoles, referred to as Nieuwkoop and Faber (NF) stages, are well documented.[21] Physiologic changes encompass multiple NF stages, which can occur at different time points, depending on environmental conditions.[1,6,17,28,36] *X. laevis* frogs primarily use lungs for respiration for the majority of their life stages. However, buccopharyngeal gas exchange mechanisms, in which respiration occurs through intake of water into the mouth, and external gills are present prior to lung development. Published literature downplays the role of buccopharyngeal respiration and gas exchange in the gills, as buccopharyngeal mechanisms are thought to have a larger role in feeding under normal environmental conditions.[8,9] Likewise, older literature states that *Xenopus* gills lack true gill filaments,[13] although recent evidence shows that gill

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filaments exist, but in smaller numbers than many other species.[22] *Xenopus* frogs also use cutaneous respiration,[14] but the role that it plays in tadpole respiration has not been studied.[29] The external gills are present during NF stages 39 to 53. The external gills undergo vascular regression during NF stages 58 to 62.[3] Lung development overlaps external gill development, and begins during NF stages 46 to 52.[21,29]

In addition to differences seen in MS222 euthanasia efficacy between adult and larval zebrafish, a recent study has shown that similar differences in efficacy exist for eugenol and hypothermic shock using rapid chilling, 2 methods of euthanasia approved for use in zebrafish.[34] Previous studies show eugenol to be an efficacious anesthetic in adult *Xenopus* frogs,[11,12,16] but its efficacy as a euthanasia solution for larval *Xenopus* is unknown. Hypothermic shock is not currently an accepted method of amphibian euthanasia,[2] but it has been suggested that its use be reconsidered.[20,31,32] Therefore, there is a need to generate evidence-based research to determine the effectiveness of this method in amphibians, specifically *X. laevis* frogs. Given the small size of tadpoles, the presence of gills, and the role played by buccopharyngeal respiration at various larval stages, eugenol and rapid chilling may be viable alternatives to MS222 for practical euthanasia of *X. laevis* tadpoles in a research setting.

The goal of this study was to identify practical and effective methods of euthanasia for *X. laevis* tadpoles at different developmental stages that include gills, buccopharyngeal respiration, and early lung development. Euthanasia is defined as an irreversible lack of movement and heartbeat. Effectiveness is defined as euthanasia in 100% of animals, 100% of the time after exposure to a euthanasia method. The authors hypothesize that MS222, eugenol and rapid chilling will all be effective methods for the euthanasia of larval *X. laevis* tadpoles at early transitional stages to lung functionality.

Materials and Methods

Animals and husbandry. Wildtype *X. laevis* tadpoles were obtained from breeder males and females originally purchased from Nasco (Saugerties, NY) and were bred inhouse by natural mating using human chorionic gonadotropin (Chorulon, Merck Animal Health, Madison, NJ). Ten females and 10 males were bred for a total of 7 successful matings. The colony was assessed semiannually for *Aeromonas hydrophila* as part of a regular environmental monitoring program. Tadpoles were housed in static 10-gallon tanks (37.8 L), filled with 4 to 6 gallons of water that was directly obtained from a recirculating system used to house adult *X. laevis* frogs. The standard water quality parameters for the system water were pH (6 to 7), conductivity (400 to 800 µS), temperature (16 to 20 °C), free and total ammonia (0 to 0.8 mg/L), nitrites (0 to 0.75 mg/L) and nitrates (0 to 45 mg/L). Animals were housed at 21.1 to 22.2 °C and had a 12:12 light:dark cycle. Animals were monitored daily for health. Tadpoles were fed Sera Micron Growth Food daily (Sera North America, Montgomeryville, PA.). Partial water changes of 6 to 10 L were completed 3 times per week and a tank change was completed once, approximately halfway through the study. Nitrates, nitrites, free and total ammonia, pH and conductivity were monitored 3 times per week, prior to water changes. The University of North Carolina at Chapel Hill IACUC approved the research protocol and research was completed in an AAALAC accredited institution. At the end of the experiment, adult frogs were donated to another laboratory on campus. All tadpoles that were not successfully euthanized during the actual experiments were humanely euthanized using MS222 at a concentration of 6 g/L, a dose that was demonstrated to be effective in this study, and compliant with institutional guidelines for the euthanasia of adult *X. laevis*.

Euthanasia solutions. Tricaine-S brand pharmaceutical-grade MS222 (Western Chemical, Ferndale, WA) and eugenol (pharmaceutical secondary standard; Sigma-Aldrich, St Louis, MO) solutions were prepared with water from the static housing tank. MS222 was buffered to a pH of 7 with sodium bicarbonate (Pentair Aquatic-Eco Systems, Apopka, FL) and both solutions were maintained at a pH of 7.0 to 7.3, as measured by a pH meter (Fisherbrand Accumet AE150 Benchtop pH Meter, Fisher Scientific, Hampton, NH). Ice was mixed with tank water in a 5:1 ice to water ratio for rapid chilling. Animals were placed in bags with 2 to 4 °C tank water to avoid direct exposure to ice, as recommended for zebrafish, and the temperature of the water in the bags was verified at 2 to 4 °C via submersion of temperature probes during testing. Recovery beakers contained tank water.

Pilot studies. Two pilot studies were completed to determine the appropriate immersion time and concentrations for the euthanasia solution for the larger study. Initially, *X. laevis* tadpoles at NF stages 43, 46, 47 and 48, confirmed by microscopic examination, were placed in 5 mL of euthanasia solution in 6-well plates containing various concentrations of either MS222 or eugenol (n = 3), or room temperature water for a control group (n = 1). Animals were placed in euthanasia solution for 60 min, then placed in room temperature tank water for a 60-min recovery period. Heartbeats were assessed at 30-min intervals during immersion in the euthanasia solution and the recovery solution. To reduce animal numbers, a modified up and down method of dosing was used to narrow down the concentration for the larger study group, based on anesthesia and euthanasia literature for amphibians and fish.[2,11,16,33,34] This approach led to the following concentrations being tested in the initial pilot: 0.25, 1, 2, 3, 4, 8,12 g/L of MS222, and 100, 250, 400, 500, 600 1000, 1500 µL/L of eugenol. Lower doses were used in morning testing sessions. Afternoon testing concentrations were determined by the data obtained earlier in the day. In addition, if significant tissue damage was noted, the corresponding concentration was not used for future time points.

To confirm the cessation of heartbeat using effective concentrations determined in the first pilot, a second pilot study was completed using 2, 4 and 6 g/L of MS222, 400 and 600 µL/L of eugenol (n = 8), and controls (n = 4) for the euthanasia of *X. laevis* tadpoles at NF stages 43, 46, 47 and 49.

Animals were placed in 2 mL of euthanasia solution in 12-well plates for 15 or 30 min then placed in 2 mL of tank water for recovery for 1 h. For both pilot studies, animals were observed for a heartbeat using a microscope (Stereo Zoom. Bauch and Lomb, Incorporated, Laval, Quebec, Canada) or an inverting microscope (CK2, Olympus Corporation of the Americas, Center Valley, PA) depending on the orientation within the well plate. Tadpoles at NF stage 43 were too small to accurately assess for a heartbeat; thus, they were assessed for movement only, using gentle touch from a pipette tip. Due to the potential for inconsistent results with this verification method, NF stage 43 was not included in the larger study.

Study methods. Tadpoles at NF stages 46, 47 and 49 were randomly assigned to either one of 3 study groups (n = 70) or a control group (n = 40). Animals were placed in 500 mL of 6 g/L MS222, 800 µL/L eugenol or 5:1 ice to water ratio rapid chilling groups or a control group of 20 animals in tank water for 15 min. Next, animals were placed in 500 mL of tank water in 1 L tempered glass beakers for recovery, where they remained for 4 h (Figure 1). Half of each group was tested in duplicate to ensure consistency between clutches.

Figure 1. Testing equipment. Each 1 L glass beaker was filled with 500 mL of euthanasia solution, a 5:1 ratio of ice to tank water or room temperature tank water for recovery. A plastic bag was used to prevent ice from maintaining direct contact with tadpoles. Thermometers measured the temperatures for the rapid chilling and control groups. Time was kept with stopwatches.

Physiologic testing. Tadpoles were assessed for movement or response. Tadpoles that were not visibly moving or were not moving when touched were assessed for the presence of a heartbeat using a microscope immediately after placement in the recovery beaker and again after 4 h. Tadpoles were placed into 12-well plates filled with 2 mL of tank water and were assessed under a dissection or inverting microscope depending on the animal's orientation within the well plate. Animals were then placed back into the recovery tank. In addition, a subset of tadpoles (n = 20) from each group that were not moving was assessed for a heartbeat at 60, 120 and 180 min to assess trends in gain or loss of cardiac function over time. Animals that were either actively moving in the beaker or began moving during attempts to collect them for visual heartbeat assessment were counted as having heartbeats.

Statistical Analysis. Fisher exact tests (SAS Visual Statistics, SAS Institute, Cary, NC) elucidated significant differences in irreversible euthanasia rates between the 3 euthanasia methods in this study. A P value of 0.05 or less indicated a significant difference.

Results

Pilot study results further developed the study design. Two pilot studies were completed to determine the optimal concentrations of MS222 and eugenol for testing in the larger study. Although MS222 at 6 g/L was not included in the initial pilot, it was used because it was midrange between 4 g/L, which was ineffective in causing cessation of heartbeats, and 8 g/L, which caused microscopic tissue damage. Eugenol concentrations of 1000 µL/L led to grossly visible dissolution of tissue, and 600 µL/L appeared to be effective in smaller volumes of water. However, the effectiveness of euthanasia during NF46 testing was variable in the large study. This timepoint was therefore repeated using a dose of 800 µL/L, and this concentration was subsequently used.

MS222 consistently causes cessation of heartbeats at all larval stages tested. To assess the efficacy of MS222 for euthanasia of *X. laevis* larvae, 70 tadpoles from each stage were immersed in 6 g/L MS222 for 15 min and each tadpole subsequently assessed for a heartbeat. None of the animals at any developmental stage had visibly detectable heartbeats after immersion in the euthanasia solution. A lack of a heartbeat continued throughout placement in a recovery tank, as observed by a subset of animals at 60, 120 and 180 min in recovery. None of the animals (n = 68 to 70) had detectable heartbeats at any time point during recovery (Figure 2). Tadpoles were removed from the study if evisceration occurred, likely caused by the breakdown of the structural integrity of the skin over time due to contact with the euthanasia solution.

Eugenol immersion causes slow and inconsistent cessation of heartbeats. The efficacy of eugenol for euthanizing larval *Xenopus* tadpoles was determined by placing tadpoles in 800 µL/L eugenol for 15 min and then assessing animals for a heartbeat. Tadpoles displayed a variable rate of heartbeat cessation after 15 min in the euthanasia solution. At NF stages 46 and 49, we saw an overall trend of the number of detectable heartbeats decreasing over time in recovery, as observed by a subset of animals (n = 20). All tadpoles at NF stage 46 and 49 (n = 68 to 70 per group) had cessation of a heartbeat after 4 h in the recovery beaker. However, at NF stage 47 (n = 68 to 69), only 88.2% of the tadpoles had cessation of heartbeats after 4 h, thus providing incomplete euthanasia for the group (Figure 2). Tadpoles were excluded from the study if evisceration occurred.

Rapid chilling fails to cause cessation of heartbeats in all larval stages tested. Animals were observed for a heartbeat after 15 min of indirect immersion in an ice bath at 2 to 4 °C and periodically in recovery containers for 4 h. A heartbeat assessment was not performed on animals that were actively moving in the container. Only 1.4% of tadpoles in the NF stage 46 group had heartbeat cessation after 15 min of cold exposure, which was consistent with what was seen after 4 h in recovery. None of the animals moved at the time of initial placement in recovery. However, many animals were moving at each hour during recovery. The NF stage 47 and 49 groups had tadpoles that were moving soon after placement in the recovery containers. All tadpoles (n = 70) retained a heartbeat or were moving at the end of 4 h in the stage 47 and 49 groups (Figure 2).

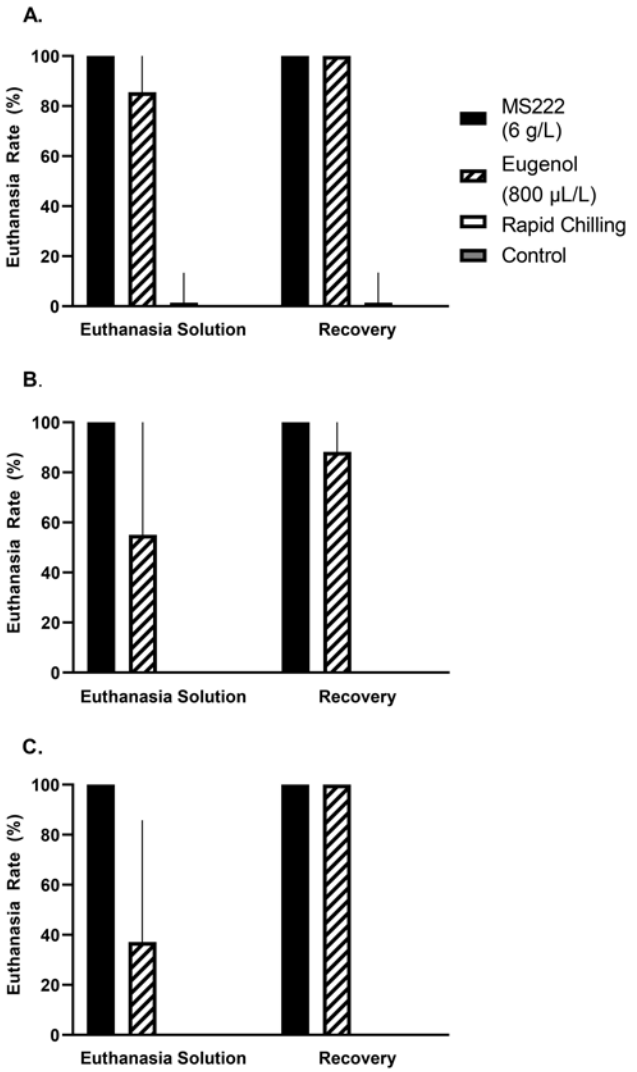

Figure 2. Percentage of *X. laevis* tadpoles euthanized after 15 min of immersion in euthanasia solution and 4 h in a recovery tank. Euthanasia is defined by a lack of movement and heartbeat. Study groups, n = 68–70. Control groups n = 40. $P < 0.0001$. (A) NF stage 46. (B) NF stage 47. (C). NF stage 49.

Discussion

This study compared the effectiveness of MS222, eugenol and rapid chilling as euthanasia methods for *X. laevis* tadpoles at transitional developmental stages of oxygen exchange. Developmental stages 46,47 and 49 represent NF stages in which external gills were present, but the lungs are still developing,[21] and their functionality is likely variable. The time points at which tadpoles achieve different developmental stages varies based on housing and environmental factors.[1,14,17,36] Therefore, the research community should address euthanasia of larval frogs in terms of developmental stages and variability at various timepoints. Because of this variability, even within a single clutch, a standardized approach to euthanasia of larval *X. laevis* tadpoles that is effective across all larval stages represents a significant technical refinement.

Overall, we found that each euthanasia method tested had a consistent effect at each of the developmental stages tested, suggesting that response to these methods does not vary markedly across the larval stages tested. Furthermore, immersion in MS222 at a concentration of 6 g/L for 15 min consistently resulted in heartbeat cessation in a timely manner in all animals tested.

Our results indicate that immersion of tadpoles in MS222 is a rapid and irreversible method of euthanasia for tadpoles at transitional respiratory stages. This outcome suggests that MS222 is an adequate primary method of euthanasia for large groups of *X. laevis* tadpoles because it meets the criteria for appropriate euthanasia for the *AVMA Guidelines for the Euthanasia of Animals* and *The Guide for the Care and Use of Laboratory Animals*.[2,19] This method is also practical in a lab setting, because it allows researchers to expeditiously and consistently euthanize large cohorts of tadpoles without the need for a secondary physical method of euthanasia, which can be distressing for personnel and given the small size of tadpoles, can potentially damage the tissue needed for experimental studies.

Our results show similar efficacy to studies that use MS222 for euthanasia of adult frogs,[35] although the timeframe for immersion used in other studies varies. The mechanism of action of MS222, like other local anesthetics, is to block voltage-gated sodium channels, leading to depression of cardiac and respiratory function.[7] Multiple studies have shown that MS222 is safe and effective in frogs as both an anesthetic and euthanasia solution.[5,15,25,35] Although MS222 use for euthanasia is practical and readily available, considerations should be taken to ensure appropriate use based on the species and life stage as well as the safety of lab personnel. One case report has shown that chronic exposure to MS222 induced reversible retinal toxicity in a lab worker.[4]

Eugenol has been described as an anesthetic in adult *X. laevis* frogs[11,16] and as a euthanasia agent in fish,[2] but has not been described as a euthanasia agent in *Xenopus*. Our pilot study revealed that on a small scale, individual setting, eugenol at a concentration of 600 µL/L was adequate to produce 100% cessation of heartbeats in larval *Xenopus*. However, once we began our study on a larger, more practical scale, this method was inadequate, and we therefore increased the dose to 800 µL/L. Eugenol at concentrations of 800 µL/L showed variable efficacy in causing cardiac arrest at 15 min in *X. laevis* tadpoles at different developmental stages. The differences seen between the small-scale pilot study and the larger study highlight the importance of completing such euthanasia research studies in both large and small scales to make better predictions of the euthanasia effects on a population.

In the NF stage 46 and 49 groups, the majority of the tadpoles had cessation of heartbeats after 15 min of immersion in eugenol, but others were immobilized yet retained a heartbeat. Over time, cessation of heartbeat did occur, and none of the tadpoles had visible heartbeat after 4 h. The trend for stage 47 showed that only 55% of the tadpoles had a cessation of heartbeat after immersion in eugenol solution, and only 88% of those animals still lacked a detectable heartbeat after 4 h in the recovery tanks. Therefore, 15-min immersion of *X. laevis* tadpoles in eugenol at a concentration of 800 µL/L is not adequate for euthanasia in a group setting.

Eugenol is a phenolic compound that acts as a local anesthetic by blocking voltage-gated sodium channels.[24] At anesthetic doses, it does not appear to be toxic in adult *X. laevis*, although its effects vary in adult *Xenopus* at different weights, with small frogs having a decrease in heart rate and oxygen saturation.[11,12] Surface area, drug metabolism, and skin permeability may all affect eugenol pharmacokinetics.[11] Although eugenol and similar compounds such as iseoeugenol and clove oil have been successfully used as an anesthetic in frogs,[11,12,33] the AVMA has only established eugenol as an acceptable euthanasia method in finfish, with the caveat of being acceptable with conditions for laboratory fish.[2]

The effectiveness of eugenol was also variable for larval zebrafish, based on dose and exposure time.[34] We also saw variation in the efficacy of eugenol euthanasia of larval tadpoles. This may have been caused by the increased surface area to body weight ratio of tadpoles compared with adults, differences in oxygen consumption between different growth stages, or the effect of water temperature on metabolism. Regardless, eugenol at the immersion time and dose tested is not consistent and rapid enough to cause adequate euthanasia for large numbers of larval tadpoles.

Our data show that rapid chilling was ineffective in euthanizing *X. laevis* tadpoles. Not only did animals fail to have cessation of heartbeats, but they also eventually regained movement over time, with some animals in stage 47 and 49 group having regained movement soon after placement in recovery tanks (Figure 2). Our findings provide evidence to suggest that rapid chilling alone is unsuitable for euthanasia in larval *X. laevis*, but this result should not necessarily be extrapolated to adult frogs or other amphibian species.

The use of cooling and freezing for anesthesia and euthanasia in amphibians and reptiles is a topic of ethical debate.[20,31,32,37] Opponents of these methods cite a lack of physiologic evidence and information gaps that would render these methods humane.[37] Proponents argue a lack of evidence of cold thermal pain during full body cooling to justify their support for use.[20] In addition, proponents argue that opponents use speculation rather than fact as well as extrapolation from mammals.[20] The 2013 version of the *AVMA Guidelines for the Euthanasia of Animals* states that hypothermia is inappropriate for amphibians unless animals are less than 4 grams and are rapidly frozen in liquid nitrogen.[2] However, unlike zebrafish, rapid chilling by immersion at temperatures of 2 to 4 °C is not specifically addressed for amphibians. A recent study on adult cane toads (*Rhinella marina*) suggested that rapid cooling, then freezing could be a humane method of euthanasia for some amphibians.[31]

Our outcome for rapid chilling is vastly different than what has been shown to be effective in zebrafish adults and fry at 14 d after fertilization.[34,38] This may be explained by the *X. laevis*' lower temperature range for laboratory housed frogs (17 to 24 °C),[14] which may make them more resistant to cold temperatures than zebrafish. The completion of a similar study using *X. tropicalis* tadpoles, which have a temperature range (24 to 25 °C) more similar to that of zebrafish (25 to 28 °C),[14,23] could potentially show rapid chilling to be effective in the *X. tropicalis* species.

To our knowledge, this is the first published study to evaluate the efficacy of rapid chilling in *X. laevis* tadpoles. Given the observations of recovery seen in this study, it may be worthwhile to further investigate the use of rapid chilling for anesthesia in *X. laevis* tadpoles in attempts to improve tadpole welfare, especially for studies in which chemical anesthesia may potentially alter study results.

The current study shows that immersion of multiple tadpoles in MS222 for 15 min at a concentration of 6 g/L is effective as a euthanasia method, as defined by lack of recovery from cessation of heartbeat. This method has significant practical applications for euthanasia of large clutches or cohorts of study animals. Although this method is useful for *X. laevis* tadpoles at developmental stages 46 to 49, further studies are needed to determine if this method can be expanded to other larval tadpole stages. In addition, this study does not account for the possible physiologic differences and specific temperature adaptations between *X. laevis* and *X. tropicalis* frogs. Similar studies should be repeated in *X. tropicalis* frogs to provide better guidance for euthanasia of tadpoles of that species.

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