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Effects of Using Tricaine Methanesulfonate and Metomidate before Euthanasia on the Contractile Properties of Rainbow Trout (*Oncorhynchus mykiss*) Myocardium

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Because many anesthetics work through depressing cell excitability, unanesthetized euthanasia has become common for research involving excitable tissues (for example muscle and nerve) to avoid these depressive effects. However, anesthetic use during euthanasia may be indicated for studies involving isolated tissues if the potential depressive effects of brief anesthetic exposure dissipate after subsequent tissue isolation, washout, and saline perfusion. We explore this here by measuring whether, when applied prior to euthanasia, standard immersion doses of 2 fish anesthetics, tricaine methanesulfonate (TMS; 100 mg/L, *n* = 6) and methyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate (metomidate, 10 mg/L, *n* = 6), have residual effects on the contractile properties (force and work output) of isolated and saline-perfused ventricular compact myocardium from rainbow trout (*Oncorhynchus mykiss*). Results suggest that direct exposure of muscle to immersion doses of TMS—but not metomidate—impairs muscle contractile performance. However, brief exposure (2 to 3 min) to either anesthetic during euthanasia only—providing that the agent is washed out prior to tissue experimentation—does not have an effect on the contractile properties of the myocardium. Therefore, the use of TMS, metomidate, and perhaps other anesthetics that depress cell excitability during euthanasia may be indicated when conducting research on isolated and rinsed tissues.

Abbreviation: TMS, tricaine methanesulfonate

Anesthesia is common practice in fish used for research and aquacultural purposes. For both applications, it is important to understand the effects of the anesthetic on the animal and tissues of interest, to ensure the validity of data and to improve animal wellbeing where possible. The efficacy of common anesthetics has been explored in many fish species.[13,18] In addition, the physiologic effects of various anesthetics have been determined in several species.[10,11,19,21] Many commonly used fish anesthetics work through depressing cell excitability[7,15]. For instance, in axons of giant squid, the tricaine moiety of tricaine methanesulfonate (TMS) binds to sodium channels and reduces depolarizing current.[7] Other common fish anesthetics, including benzocaine and eugenol-based compounds, are thought to act through similar mechanisms, depressing cell excitability.[1,4,15,17] The anesthetic methyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate (metomidate), a soft etomidate analog, acts in fish by binding to $GABA_A$ receptors in the brain, thus enhancing the receptor's function and generating the hypnotic and immobilizing effects of etomidate and its analogs.[8,17] Due to these effects, metomidate has been explored for its potential use in the pet trade as a sedative to reduce transportation stress.[11]

Given the mechanisms through which many of these anesthetics act, their use typically is contraindicated in research that relies on the excitable nature of tissues such as muscle and nerve. For example, TMS dose-dependently reduces twitch force in isolated myocardium of Chinook salmon (*Oncorhynchus tshawytscha*).[10] Similarly, prolonged immersion of spinally transected rainbow trout (*O. mykiss*) in TMS leads to reductions in heart rate and blood pressure that may indicate a loss of myocardial function.[19] However, if these affects can be effectively and rapidly reversed by saline rinsing of isolated excitable tissues, then researchers might consider refining euthanasia of fish to include anesthetic use under appropriate circumstances. The *CCAC Guidelines on Euthanasia of Animals Used in Science*[5] considers physical methods of fish euthanasia conditionally acceptable only when scientific justification for the lack of anesthesia is provided and when operators are well trained.[5] Physical methods of euthanasia, when provided as the sole method, appear more prone to error that could result in stress or pain than when used in conjunction with anesthetics, and personnel being trained in physical euthanasia likely would benefit from having the fish sedated or anesthetized to reduce movement of the fish. In addition, the 2013 edition of the *AVMA Guidelines for the Euthanasia of Animals*[2] further notes that physical means of fish euthanasia require expert training, again suggesting an increased possibility for error.[2]

Here we tested whether immersion exposure of a commonly studied fish species, rainbow trout, to 2 fish anesthetics, metomidate and TMS, prior to euthanasia affected the mechanical properties of ventricular compact myocardium that subsequently was isolated and saline-rinsed prior to experimentation. TMS is often used in research; this agent is known to act peripherally and thus was expected to directly affect the tissue of interest. Metomidate appears to be more potent than is TMS (that is, a lower dose of metomidate achieves similar planes of anesthesia[10,20]), has stress-suppressing properties,[16,20] and may exert most of its effects centrally, thus minimally affecting peripheral

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tissues of interest. In determining whether the refinement of euthanasia by adding anesthesia may be indicated in various studies, we hypothesized that compact ventricular tissue from fish anesthetized prior to euthanasia and exposed to subsequent anesthetic-free saline rinses would not differ in contractile properties compared with muscle from fish that were not anesthetized during euthanasia. Furthermore, we hypothesized that any effects on contractile properties would be less after or during exposure to metomidate than to TMS.

Materials and Methods

All procedures were approved by the University of Calgary animal care committee following the Canadian Council on Animal Care guidelines. Juvenile, triploid rainbow trout (n = 18; male and female; weight, 50.3 ± 2.4 g) were obtained from Sam Livingston Fish Hatchery (Calgary, Alberta, Canada). Triploidy was confirmed by measuring the major axis of the erythrocytes.[3] Prior to experimentation, fish were held in 8 rectangular, 60-L fiberglass tanks (Kahlsico International, El Cajon, CA), supplied with air-saturated, dechlorinated city tap water and a maximal density of 3 fish per tank to avoid crowding. The tank system was configured for recirculation and supplied with water flowing through a limestone gravel biofilter and inline chiller (model WM-500, Aquarium Systems, East Lake, OH). Fish were monitored daily for signs of stress, and water chemistry (ammonia, 0.5 mg/L [average]; nitrite, less than 0.0 mg/L (undetectable); nitrate, less than 0.0 mg/L (undetectable), pH 8; and $CaCO_3$, 140 mg/L) was monitored routinely and confirmed to be of high quality throughout the study. The pathogen status of the trout was not monitored during the course of the experiment; however, fish appeared healthy and manifested no overt signs of stress. Trout were fed approximately 1% body weight of commercial trout pellets daily (Classic Sinking Fish Feed for Salmonids, 5 mm feed crushed to 2 mm, Martin Mills, Ontario, Canada) and were exposed to a 12:12-h light:dark period. Temperature was held at 11 to 13 °C.

Anesthetics and euthanasia. Fish were euthanized either with anesthetic present (TMS or metomidate) or without (control). Fish undergoing euthanasia with anesthetic treatment were exposed to either TMS (100 mg/L; Syndel Laboratories, Qualicum Beach, British Columbia, Canada) buffered to pH 7 or to metomidate at pH 8 (10 mg/L; Aquacalm, Syndel laboratories, Qualicum Beach, BC, Canada). The doses selected represent standard dosage for rapid induction (less than 3 min) of anesthesia for rainbow trout.[20] Fish were removed from the holding tank by using a dip net and placed into a 3-L tank filled with water from the holding tank and containing either TMS, metomidate, or no anesthetic. Anesthetized fish (n = 6 per group) were held in this tank until they lost their righting ability and fin movements had ceased (that is, 2 to 3 min), defined as stage 2 anesthesia.[14] Control fish (n = 6) were handled in the same manner and were kept in the 3-L tank of holding system water (without anesthetic) for 2 to 3 min. The fish then were quickly removed from the tank, placed in ventral recumbency on a flat surface, and euthanized through a sharp blow directly over the brain with a wooden baton and followed immediately by pithing of the brain and spinal cord, as indicated in the AVMA guidelines.[2] All euthanasia procedures were performed by trained and experienced personnel.

Tissue preparation. After euthanasia, the hearts were removed and placed in chilled, anesthetic-free saline (NaCl, 132 mM; KCl, 2.6 mM; $CaCl_2$, 2.7 mM; $MgSO_4$, 1.0 mM; NaH_2PO_4, 1.0 mM; glucose, 10.0 mM; HEPES, 10 mM; pH, 7.8). Hearts then were cut in half by using a razor blade and pinned open in a 20-mL dissection dish. The dish was flushed with chilled saline 2 or 3 times to remove blood, with the saline stream directed over the heart tissue to ensure rinsing. A strip of compact myocardium from the ventricle was isolated, and ties of 6-0 silk suture were secured on either end. The ties were attached to a servomotor (model 350, Cambridge Technology, Bedford, MA) at one end and to a force transducer (model 400a, Aurora Scientific, Aurora, Ontario, Canada) at the other. Preparations were then bathed in anesthetic-free saline at 12 °C in a temperature-controlled chamber. To maintain air saturation, the saline in the chamber was bubbled with atmospheric air. Preparations remained in anesthetic-free saline for the duration of dissection (20 to 30 min) and then in the chamber for the remainder of the experiment (about 30 min for setting baseline parameters including stimulus voltage and muscle length and an additional 30 to 60 min for measures of contractile performance). Therefore, after euthanasia, samples were exposed to nonanesthetic saline for about 60 min prior to experimentation, during which the saline was changed 2 or 3 times, after which the samples were placed in an approximately 35-mL reservoir of saline during experiments.

Measures of contractile performance. To test the effects of anesthetics used in euthanasia, we assessed 4 measures of contractile performance, each reflecting different aspects of the excitation and contractile capacity of the muscles. These included the ability to perform mechanical work, maximal isometric force production, rates of force rise during activation and force fall during relaxation, and the maximal rate at which the muscle could contract consistently with repeated stimulation. Experiments were controlled and data collected by using software custom-written by using LabView (version 6.1, National Instruments, Austin, TX) and a 12-bit analog-to-digital converter card (PCI MIO 16E-4, National Instruments).

To stimulate the muscle and elicit contractions, current was passed between 2 platinum plates placed on each side of the muscle. A stimulator (model SD9J, Astro-med Grass Instruments Division, Isabelle Brossard, Quebec, Canada) connected to the plates was used to control the duration (1-ms square pulse) and voltage of the stimulations. To ensure that the preparations were fully activated, the voltage was set to approximately double that required to evoke maximal force production by using a single pulse.

To measure the ability of the muscles to do work, as would occur during repeated contractions of the heart, the work–loop method was used in which cyclical strain (lengthening and shortening) was imposed on the muscle by the servomotor, and stimulation was applied at an appropriate phase of the strain cycle so that net work output was maximized.[9,22,23]The servomotor imposed strain on the preparation in a sinusoidal pattern at an amplitude of 10% peak-to-peak of the muscle resting length. The preparations were stimulated once during each strain cycle, with the onset of stimulation occurring at 10% of the period of the imposed strain cycle. In this way, the muscles produced force primarily during the shortening portion of the strain cycle and were relaxed during the lengthening portion, as occurs in a beating heart. Before measurements of work and force began, the resting length of the preparation was adjusted to produce maximal net work output. To this end, the preparations were systematically lengthened in 0.1-mm increments; at each length, a 5-cycle series of strain and contractions at a cycling frequency of 0.75 Hz (45 contractions per minute) was imposed on the muscle. The length that resulted in maximal net work output was used for the remainder of experiments.

Once the optimal length was determined, heart preparations underwent 10 cycles of strain and contraction at a cycling

frequency of 0.75 Hz. Work output from the last cycle was used for the analysis, where force and work output had stabilized (the average difference in net work output between cycles 9 and 10 was only 3.3%). Work done by the muscle during the shortening portion of the strain cycle (shortening work) and that required to lengthen the muscle (lengthening work), were measured as the integral of force with respect to muscle length change. The net work output over a complete cycle was calculated as the difference between the shortening and lengthening work. Work was standardized to the mass of each muscle preparation (J/kg).

Isometric twitch force then was measured at the muscle length found to be optimum for work output. The optimum length for work output and developed isometric twitch force are similar in the compact ventricular myocardium of rainbow trout.[5] Developed isometric twitch force was calculated as the difference between the maximal twitch force and the resting, passive force. Force was standardized to the cross-sectional area of the preparation (N/m^2). Cross-sectional area was determined for each preparation by using the measured muscle length and mass and assuming a muscle density of 1.05 g/cm^3. In addition, we assessed 2 measures of twitch kinetics: the time required for force to rise from 10% to 90% of the maximal isometric twitch force during contraction and the time required for force to fall from 90% to 10% during relaxation.

Preparations then underwent isometric contraction at increasing stimulation frequencies to determine whether TMS and metomidate affected the frequency at which the muscle became refractory. The muscle was considered refractory when the developed force of successive twitches within the final 10 twitches of a series differed by more than 10%. Muscle preparations were exposed to 20 successive isometric twitches at different rates of stimulation; the lowest rate was 1.0 Hz, which was then increased by 0.1 Hz for each successive series of contractions.

Finally, myocardium preparations were exposed directly to the same doses of buffered TMS (100 mg/L; $n = 6$) and metomidate (10 mg/L; $n = 6$) as used during immersion anesthesia, and work and force were measured. This experiment was designed to demonstrate whether these drugs had a direct effect on myocardial contractile performance and therefore whether brief exposure prior to euthanasia followed by saline rinses was effective at eliminating or avoiding this effect. Myocardial preparations were exposed to the same anesthetic that was used during euthanasia of each individual fish to avoid any effect of drug interactions. Work and developed twitch force were measured after 5 min of anesthetic exposure with no subsequent saline rinse (that is, preparations remained immersed in saline containing anesthetic during measurements).

After all experiments, myocardial preparations were removed from the bath, trimmed to remove obviously dead tissue and the tissue located outside of the ties, blotted on filter paper (Whatman, Springfield Mill, United Kingdom) to remove surface saline, and weighed on a balance (model MT5, Mettler–Toledo, Mississauga, Ontario, Canada).

Analysis. Each measure of contractile performance was compared between the 3 methods of euthanasia (TMS, metomidate, control) to determine whether anesthetic use during euthanasia had any residual effect on the contractile properties of the myocardium. Statistical comparisons were made by using one-way ANOVA, and a P value less than 0.05 was considered significant. In the analysis of relaxation kinetics (time from 90% to 10% developed force) and refractory heart rate, the assumption of equal variance was violated (Brown Forsythe, $P < 0.05$). Therefore, nonparametric Kruskal–Wallis tests were performed on rank-transformed data from these analyses. To assess the effects of direct anesthetic exposure on work output and developed twitch force, paired t tests were used to compare performance in anesthetic-free saline and after 5 min of anesthetic exposure. All statistical analyses were completed by using Sigmastat (Sigmaplot version 13, Systat Software, San Jose, CA**).**

Results

None of the measures of contractile performance differed significantly between any of the 3 euthanasia treatments: developed twitch force, $P = 0.28$ (Figure 1); 10% to 90% twitch kinetics, $P = 0.212$; 90% to 10% twitch kinetics, $P = 0.157$ (Figure 2); net work, $P = 0.381$; shortening work, $P = 0.243$; lengthening work $P = 0.435$ (Figure 3); and the stimulus rate at which the preparations became refractory, $P = 0.677$ (Figure 4).

Direct exposure to buffered TMS significantly affected both work and twitch force ($P < 0.001$ both comparisons; Figure 5). After 5 min of exposure to buffered TMS, all preparations failed to produce positive net work output. Conversely, direct exposure to metomidate did not impair either work output ($P = 0.15$) or twitch force ($P = 0.12$; Figure 5).

Discussion

Substantial negative inotropic effects of TMS have previously been demonstrated in fish myocardium,[6] as occurred in the present study when buffered TMS was applied directly to isolated muscle segments from rainbow trout (Figure 5). Conversely, when ventricular strips were directly exposed to metomidate, work output and twitch force were not significantly reduced, although the averages were slightly less than those of controls (Figure 5). This trend may reflect the brief (5 min) exposure to metomidate, given that a small drop in twitch force has been observed in ventricular strips exposed directly to metomidate at the same dose as we used here but over a longer time period.[10] Regardless, any negative inotropy associated with TMS and metomidate exposure during euthanasia appears to be relatively easily reversible or is perhaps not present providing that the immersion in anesthetic solution is brief. The contractile properties of isolated and saline rinsed myocardium from trout anesthetized with buffered TMS or metomidate prior to euthanasia did not differ compared with control values (Figures 1 to 4). Therefore, a simple saline rinse was sufficient to reverse any effects these anesthetics might have on the excitability and contractile properties of myocardium after exposure of the fish prior to euthanasia; alternatively, tissue exposure to the anesthetic agents during immersion was sufficiently brief that it did not alter contractility.

As noted, tricaine acts to depress cell excitability by reducing the inward Na^+ current.[1,4,7] This effect is likely a result of changes to the ion channel or its gating properties on binding to tricaine. The results noted here, where the effect of tricaine is readily and rapidly reversed by saline rinse, may suggest a relatively weak association of tricaine to the Na^+ ion channel of rainbow trout myocardium. Although the binding affinity of tricaine to Na^+ channels of the myocardium in rainbow trout has not been examined, tricaine has been shown to rapidly clear from the blood after the placement of fish in anesthetic-free water,[6,12] supporting the selection of TMS as an appropriate anesthetic for use prior to euthanasia when isolated tissues will be rinsed, particularly when exposure is brief. Furthermore, at the doses used, effects on the contractile performance of myocardium were either absent or minimal when preparations were exposed directly to metomidate compared with TMS. This finding may imply that metomidate's action in vivo targets the CNS more

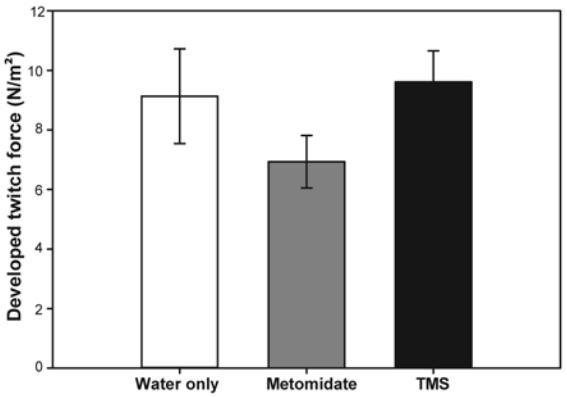

Figure 1. Developed isometric twitch force of saline-rinsed myocardium after immersion exposure of the fish to buffered TMS, metomidate, or holding-tank water (nonanesthetic control) before euthanasia. Data are given as mean ± SEM (n = 6).

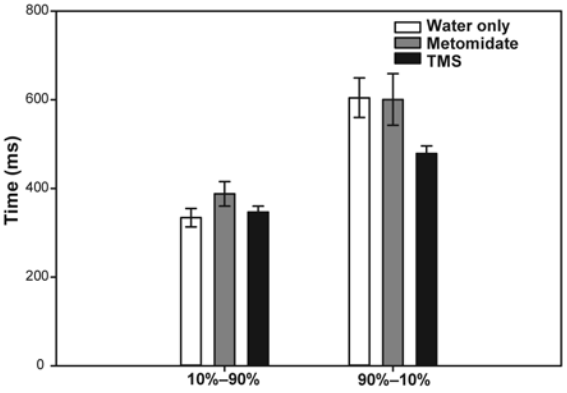

Figure 2. Time required for force to rise from 10% to 90% of the maximal developed twitch force during isometric contraction and time to fall from 90% to 10% during isometric relaxation of saline-rinsed myocardium after immersion exposure of the fish to buffered TMS, metomidate, or holding-tank water (nonanesthetic control) before euthanasia. Data are given as mean ± SEM (n = 6).

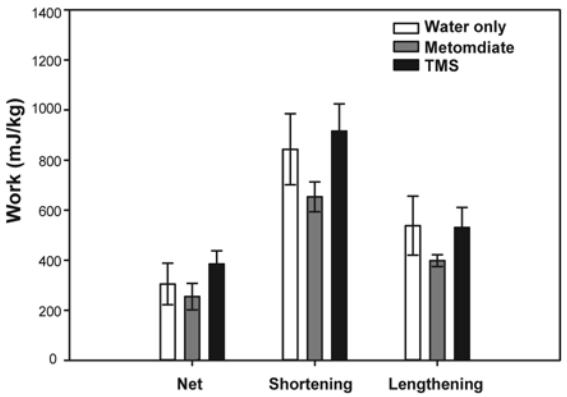

Figure 3. Mass-specific work output (net, shortening, and lengthening) of saline-rinsed myocardium after immersion exposure of the fish to buffered TMS, metomidate, or holding-tank water (nonanesthetic control) before euthanasia. Strain, 10%; cycle frequency, 0.75 Hz; and stimulation phase, 10%. Data are given as mean ± SEM (n = 6).

potently than muscle itself. As a result, metomidate appears preferable to TMS as an anesthetic for use in studies that target excitable tissues.

Both TMS and metomidate were applied through whole-animal immersion and for only brief periods. Therefore, the

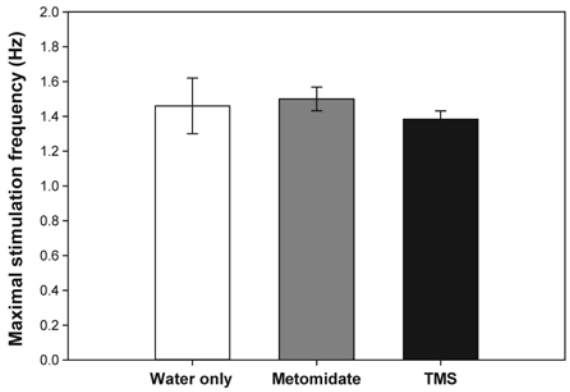

Figure 4. Maximal rate at which saline-rinsed myocardium preparations could be stimulated before becoming refractory after immersion exposure of the fish to buffered TMS, metomidate, or holding-tank water (nonanesthetic control) prior to euthanasia. Data are given as mean ± SEM (n = 6).

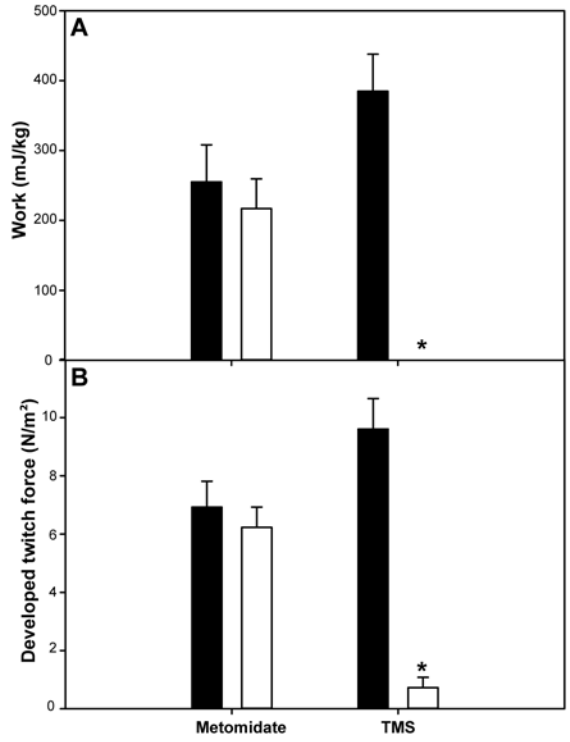

Figure 5. (A) Work output and (B) developed isometric twitch force before (solid bars) and 5 min after (open bars) direct exposure of myocardium to the anesthetics buffered TMS (100 mg/L) or metomidate (10 mg/L). Myocardium exposed directly to TMS failed to produce positive net work output in all preparations. Data are given as mean ± SEM (n = 6). *, Value is significantly ($P < 0.05$) different after anesthetic exposure.

dose experienced by the myocardium in vivo is unknown. Plasma levels of TMS after 2 min of exposure in rainbow trout were about 75% of immersion levels.[12] Exposure of the heart to anesthetic that is entering the circulation by way of the gills would likely precede exposure of other tissues, and the time course would be tissue-dependent. Therefore, although the results presented here likely reflect an extreme for tissue exposure, the sensitivity of other tissues to anesthetic exposure may differ from that of the heart. Further investigation is required to understand how other tissues may react to anesthetic exposure prior to euthanasia, even under the same exposure regimen as we used here. In addition, how completely or quickly tissues

might recover from sustained or higher doses of these anesthetics remains unknown. Future research should explore the relationship between dose, anesthetic agent, and duration of immersion, because these factors may influence the residual effects on isolated tissue.

In summary, brief exposure of rainbow trout to the immersion anesthetics TMS and metomidate before euthanasia appeared to not influence the contractile properties of isolated and saline rinsed myocardium. Therefore, studies involving the contractile properties of isolated and saline-perfused cardiac muscle from fish might be refined to include the use of the anesthetics TMS or metomidate before euthanasia. Anesthetic use enabled more secure physical restraint of the fish, thereby increasing control during physical euthanasia methods, and was considered a considerable improvement in achieving consistency regarding the application of effective euthanasia. In light of the response of myocardium to direct exposure to these anesthetics,[10] metomidate appears less likely to affect cardiac muscle performance. Both TMS and metomidate are efficacious in many fish and amphibian species, and many of the mechanisms of depressing cell excitability in myocardium are likely shared with other excitable tissues. As such, our current results may be broadly applicable to other species, other types of excitable tissues (skeletal muscles and nerves), and other anesthetics. However, the present study was limited to assessing the effects on ventricular myocardium from rainbow trout and only after brief exposure of fish in an immersion bath.

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