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Comparison of Tricaine Methanesulfonate (MS-222) and Alfaxalone Anesthesia in Bluegill Fish (*Lepomis macrochirus*)

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Bluegill fish (*Lepomis macrochirus*) are a popular sportfish across North America. Research involving bluegill has focused mainly on locomotion, environmental monitoring, bioaccumulation, and toxicology. With fish becoming more popular research models, bluegill use may increase. Consideration for sedation and anesthesia in bluegill is lacking. MS-222 is a commonly used anesthetic in fish that requires a 21-d washout period before entry into the food chain. Other, safer options for anesthesia should be available. In this study, we first determined a suitable MS-222 dose for general anesthesia, then compared it with 2 different concentrations of alfaxalone (5 and 10 mg/L). Both concentrations of alfaxalone were adequate to reach the desired anesthetic plane, although time to effect was dose-dependent and longer in these groups when compared with MS-222. Time to recovery was also prolonged in both alfaxalone groups compared with the MS-222 group. We also assessed anesthetic degradation in the water bath over time. In this study, we show that sedation with alfaxalone at 5 and 10 mg/L is just as effective as MS-222 with no degradation of either anesthetic over the time measured.

Abbreviations and Acronyms: LORR, loss of righting reflex; LOSR, loss of startle response; LOTR, loss of tactile response; MS-222, tricaine methanesulfonate; OM, opercular movement; RONS, return of normal swim; ROSR, return of righting reflex

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Introduction

Bluegill fish (*Lepomis macrochirus*) are a popular sportfish across North America. Often called brim or bream, they are a sunfish in the family Centrarchidae. Other sunfish in this family include largemouth bass, smallmouth bass, and crappie.[25,29] Bluegill have been used to study locomotion and swim dynamics,[6,38] behavior,[7,11] toxicology,[13] environmental monitoring[4,21] and bioaccumulation[18,26] in their natural environment. As their use in research increases, appropriate anesthesia protocols must be developed that reduce stress during handling and confinement and provide analgesia when painful procedures are performed, all of which ensure the welfare of the fish.[40] Although sedation is often used during transport, sample collection, tagging, and veterinary procedures for fish, anesthetic protocols are lacking for bluegill.[40]

Sedation reduces the overall response to external stimuli while general anesthesia suppresses the CNS to achieve 3 basic components: amnesia, unconsciousness, and lack of response to noxious stimuli.[1,24,37] Immersion in anesthetic agent is equivalent to inhalational anesthesia in mammals and is the most common anesthetic technique used in several fish species.[10,20,24] The anesthetic agent is absorbed through the gills to enter the bloodstream. Other methods, such as intravenous, intraperitoneal, and intramuscular administration, are not practical in smaller fish species due to their size and are also not practical in large-scale operations.[20] The ideal anesthetic agent should provide rapid, smooth, and consistent induction while providing immobilization that lasts long enough to perform the desired procedures. Recovery should be rapid, which is important when working under field conditions. The anesthetic should have a wide margin of safety where the toxic dose greatly exceeds the therapeutic dose.[10]

Tricaine methanesulfonate (MS-222) is a local anesthetic, but when used in an immersion bath, can provide general anesthesia in fish.[20] MS-222 blocks sodium ions from entering cells, preventing further transmission of action potentials and pain signals. MS-222 is commonly used for fish sedation, is the only anesthetic approved by the FDA for use in fish as food in the United States, and requires a 21-d washout period.[34] This approval is restricted to 4 families: Ictaluridae, Salmonidae, Esocidae, and Percidae. The use of MS-222 in bluegill is restricted if unable to isolate the fish during this washout period. Many sport fish producers and researchers are unable to isolate the exposed fish for this washout period, and they often elect to euthanize instead.[30] MS-222 can also possess environmental and human health hazards and must be disposed according to local, state, and federal regulations.[32] Reported side effects in animals include respiratory acidosis, cardiac depression and failure, and death.[8,20,40] Increased blood glucose, plasma cortisol, and lactate may also occur.[8,40] Solutions of MS-222 are acidic and must be buffered to a neutral pH prior to use.[30] These factors can present challenges when

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conducting studies in the bluegill's natural environment that require anesthesia.

Alfaxalone is a neuroactive steroid that targets gamma aminobutyric acid (GABA) receptors, modulating chloride ion transport to produce sedation and anesthesia and is designated as a schedule IV controlled substance in the United States.[33,42] Alfaxalone has been studied and shown efficacious in several other fish species, including koi, oscar fish, zebrafish, goldfish, rainbow trout, black spot barbs, and peacock cichlids.[3,5,14,22,27,30,41] Our laboratory recently found that in zebrafish alfaxalone did not provide a surgical plane of anesthesia in contrast to other reports.[14,36] Reported side effects include apnea with intravenous administration, cardiac depression, and rapid arousal.[42] Increases in lactic acid were reported in oscar fish when immersed in 5 mg/L alfaxalone.[5] Alfaxalone is FDA-approved for use in dogs and cats, and it is FDA indexed for use in many other species, but not for any fish species.[35] Although use is currently restricted in any food and food-producing animal, a recent study found that alfaxalone residues were 100% cleared from all tissues 36 h after exposure, which is significantly shorter than those reported in other fish studies.[23,28,30,39] These studies suggest alfaxalone could be valuable as an option for use in aquatic species that may enter the food supply or where MS-222 use is restricted.

Other possible methods and compounds for sedation for nonpainful procedures in fish include gradual cooling, isoeugenol, and metomidate hydrochloride, but MS-222 remains the primary choice for providing a surgical plane of anesthesia.[8] Many of these agents, including MS-222, are not readily available in the clinical setting, and, if available, may not be pharmaceutical grade. In the United States, alfaxalone is a schedule IV controlled substance, which could limit its applicability in the field.[33] However, reliable anesthetic protocols are necessary for animal welfare and make it possible to expand experimental procedures available for use in this species while hopefully reducing the number euthanized each year for research purposes.[40]

The concentration of anesthetics in immersion baths could dissipate with use and time. MS-222 has been reported to degrade in the presence of light.[20] There are conflicting reports concerning degradation over time under various storage conditions, however no information on decreasing concentrations with repeated fish exposure is available for use in bluegill.[19,32] To our knowledge, alfaxalone degradation over time has not been reported.

The objective of this study was to investigate the efficacy of 2 doses of alfaxalone in bluegill in producing a surgical plane of anesthesia. We hypothesized that induction and maintenance with either low-dose (5 mg/L) or high-dose (10 mg/L) alfaxalone would be as effective as MS-222 in reaching a surgical plane, would provide faster induction but a prolonged, dose-dependent recovery, and the efficacy of each anesthetic concentration tested would degrade over the testing period.

Materials and Methods

Humane care and use of animals. Research was conducted under an IACUC approved protocol in compliance with federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by AAALAC International and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Animals and housing. Adult bluegill (n = 63) weighing between 20 and 67 g were purchased (Kurtz Fish Farm, Elverson, PA) and housed in custom-built, flow-through aquaculture tanks (flow rate 2 ± 0.4 L/min) with the water temperature maintained at 23 ± 1°C. Bluegill were housed in a stocking density of one fish per 3.6 L. Overhead, full-spectrum LED lighting was set on a 14-h light/10-h dark photoperiod. Water quality parameters were maintained as follows: dissolved oxygen 60% to 100% saturation; pH 7.4 ± 0.3; alkalinity 110 to 180 mg/L as $CaCO_3$; hardness 150 to 210 mg/L as $CaCO_3$; conductivity 585 ± 10 µS/cm; total ammonia less than 0.1 mg/L as NH_3. Bluegill were fed 3 times a day, 2 feedings of finfish starter pellets (Zeigler Bros, Gardners, PA), and one feeding of frozen brine shrimp (Brine Shrimp Direct, Ogden, UT). On weekends, bluegill were fed twice with one feeding of finfish starter pellets and one feeding of frozen brine shrimp. Tanks also contained artificial plants to provide additional environmental enrichment.

Parameters defined. The authors extrapolated the methods and design recently reported for assessing anesthetic depth and recovery in zebrafish.[36] Loss of righting reflex (LORR) was defined as bluegill no longer able to maintain equilibrium and rotated at least 90°. After LORR, loss of startle response (LOSR), or response to sudden noises or vibrations, was assessed by tapping the side of the tank and watching for a response. If there was a response, the process was repeated every 15 to 30 s (not timed, based on observation) until LOSR was achieved. After LOSR was confirmed, loss of tactile response (LOTR) was assessed. LOTR assessed response to a noxious stimulus. To generate the noxious stimulus, the observer applied a moderate firm pinch to the caudal fin with a pair of blunt forceps (SS biology tweezers, anti-acid, World Precision Instruments, Sarasota, FL)[36] (Figure 1). Fish were thought to have LOTR when there was no response after 2 applications of the noxious stimulus. Opercular movement (OM) was assessed in real time by the observer, but movements per set period of time (that is, breaths per minute) were not assessed due to assessment of other parameters simultaneously. Categories for OM are defined in Table 1. For this study, the surgical plane of anesthesia (stage III, plane 2) was defined as achieving all of the following: LORR, LOSR, LOTR, and decreased OM. We considered this period the induction phase. The maintenance phase began immediately after LOTR was confirmed. Return to self-righting reflex was defined as the ability to stay mostly upright, not wobbling more than 45° to either side, and the ability to self-correct. Bluegill were scored as fully recovered when normal nonimpaired swimming and normal behavior were returned. The same individual performed the observations for the entire study for consistency in measurements; however, the observer was not blinded to the anesthetic groups.

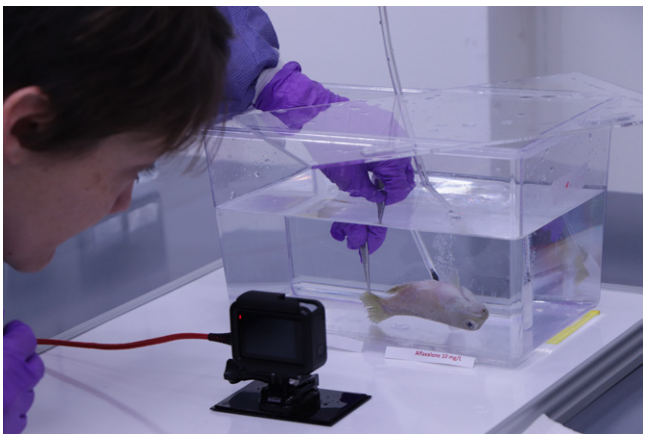

Figure 1. Tactile response test using blunt forceps to apply the noxious stimulus to the caudal fin.

Table 1. OM rate assessed during immersion

Category	Rate
0	Normal
1	Decreased
2	Shallow
3	Rare
4	Absent

1, OM slowed; 2, distance of OM decreased; 3, OM greater than 15 s between movements.

MS-222 dose optimization pilot. Bluegill were randomly assigned to 1 of 3 groups (n = 3 per group, no sex preference). The following concentrations were selected to optimize the best dose for use in the comparison study: group 1 immersed in 100 mg/L, group 2 in 150 mg/L, and group 3 in 200 mg/L. MS-222 (Sigma-Aldrich, St. Louis, MO) was added directly to the anesthetic tank filled with 4 L of water obtained from the fish culture water line. Solutions were buffered to pH 7.3 ± 0.3 (sodium bicarbonate, Sigma-Aldrich, St. Louis, MO; Pocket Pro pH tester, Hach, Loveland, CO). Fish were immersed individually in the anesthetic solution. Times to LORR, LOSR, and LOTR were recorded. Time to changes in OM were also recorded. Once LOTR was confirmed, fish remained in the anesthetic bath for approximately 1 min after loss of tactile response. Fish were then transferred to a recovery tank void of anesthetics. Time to return of righting reflex and normal swim were recorded. Once recovered, fish were returned to their home tank.

Comparison experimental design. Bluegill were randomly assigned into 1 of 3 groups (n = 18, no sex preference): MS-222 (group 1), low-dose alfaxalone (group 2), and high-dose alfaxalone (group 3). MS-222 was prepared at 200 mg/L in 4 L of fresh fish culture water and stirred to mix. This dose was based on the pilot study outlined above. Low-dose alfaxalone (5 mg/L) and high-dose alfaxalone (10 mg/L) were prepared by adding the appropriate amount of stock solution (Alfaxan multidose at 10 mg/mL; Jurox, North Kansas City, MO) to 4 L of fresh fish culture water and stirring to mix. The alfaxalone concentrations were selected based on previous studies.[3,5,22,27,30,36,41] Static tanks were prepared at the same time as the anesthetic tanks and were used for measuring concentration over time. All anesthetic and static tanks were freshly prepared immediately before the test, were buffered to pH 7.3 ± 0.3, and contained one aeration stone. Water in the tanks was changed after 9 fish. The recovery tank contained only fresh fish culture water that was replaced after each fish. Bluegill were fasted 24 h prior to anesthesia.

Induction and maintenance of anesthesia. The tank setup reported by Weaver and colleagues was used regularly in our laboratory and is the same setup described here.[36] The anesthetic immersion tank was placed in a designated area in front of a digital camera (GoPro model SPTM1; GoPro, San Mateo, CA) that recorded the movements for each fish while immersed in anesthetic (Figure 2A). Fish were exposed individually. Time to LORR, LOSR, LOTR, and changes in OM were recorded. We started with the MS-222 group. The first 4 fish remained in the immersion tank for approximately 1 min after LOTR. Corneal opacities were appreciated in recovery in each of these fish, so the exposure time was shortened to approximately 15 s after LOTR for all remaining fish, regardless of group.

Anesthetic recovery. The recovery tank setup was similar to that reported by Weaver and colleagues with the exception of the shoaling videos.[36] Bluegill do not express shoaling behavior, so these videos were not played during recovery. The recovery tank was placed in a designated area in front of a separate digital camera (Basler model acA1920-155um; Basler, Exton, PA) (Figure 2A). Movements during recovery were captured by this camera and analyzed with EthoVision XT software (Noldus, Leesburg, VA) (Figure 2B). After the anesthetic exposure, fish were immediately moved to the recovery tank and remained there for 10 min regardless of actual recovery time. Times to return of self-righting reflex and normal swim were recorded. The water in the recovery tank was replaced after each fish to avoid exposure to any possible anesthetic residue from previous fish that could prolong recovery. The aeration stone was not placed in the recovery tank, as the bubbles generated would interfere with the movement tracking software. Recovery for several fish in both alfaxalone groups was longer than 10 min. These fish were placed in a secondary tank with an aeration stone until fully recovered. All fish were returned to their home tank when fully recovered. After recovery, animals were euthanized in 500 mg/L MS-222 buffered to pH 7.3 ± 0.3 for a minimum of 30 min in accordance with the AVMA Guidelines for the Euthanasia of Animals (2020 edition).[2] After euthanasia, fish were blotted dry with paper towels and weighed (Mettler Toledo analytical chemistry scale, model XP204; Mettler Toledo, Columbus, OH).

Anesthetic concentration over time. Samples were collected from the static control tank (no fish exposure) and the

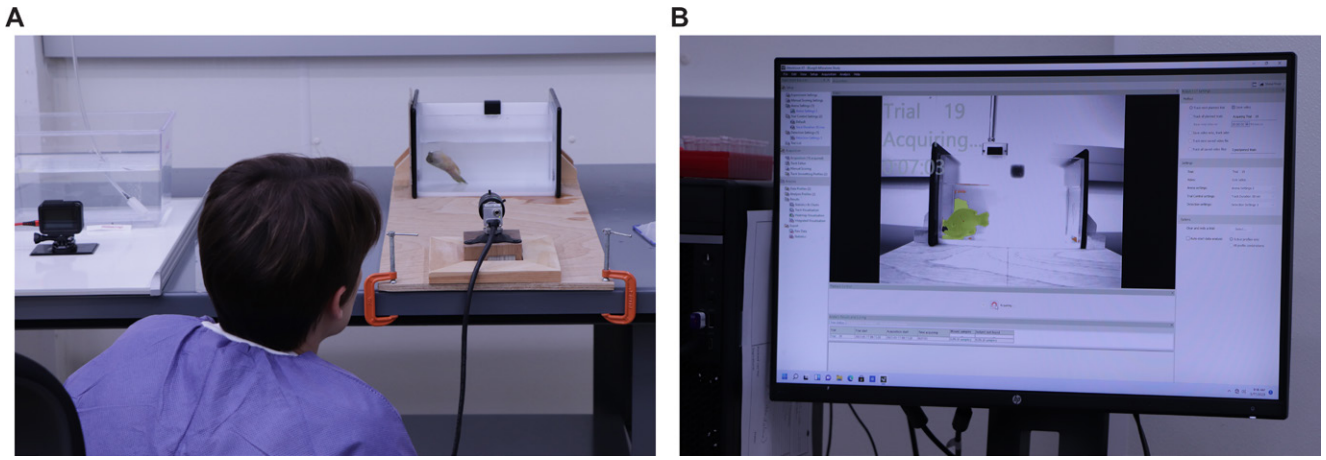

Figure 2. Experimental setup. (A) Overview showing the anesthesia tank on the left and the recovery tank on the right. The bluegill in the recovery tank has not yet regained righting reflex. (B) Image capture computer using EthoVision XT to track movement while in recovery tank.

3 different anesthetic immersion tanks at the following timepoints: baseline, after second fish, after fourth fish, after sixth fish, and after eighth fish. The samples were placed in a −80 °C freezer and stored until analysis. Anesthetic concentrations were quantified by liquid chromatography–tandem MS using a Sciex 5500 triple quadrupole mass spectrometer in line with a Shimadzu Prominence UPLC system. Sulfamethoxazole was used as an internal standard. Analytes were detected by multiple reaction monitoring mode using the following m/z transitions: MS-222 (166.1 → 75.3), alfaxalone (333.2 → 297.2), and sulfamethoxazole (254.1 → 155.9). Compound-specific parameters were as follows: alfaxalone (declustering potential [DP] = 146, collision energy [CE] = 40, collision exit potential [CXP] = 12), MS-22 (DP = 101, CE = 42, CXP = 36), sulfamethoxazole (DP = 76, CE = 23, CXP = 10). The CE was deoptimized for alfaxalone to ensure similar responses over the concentration range of the standard curve. Mass spectrometry source parameters were curtain gas (35), electrode voltage (5500 V), temperature (500° C), source gas 1 (40), and source gas 2 (50). Analytes were separated using a Hydro-RP column (30 × 2 mm, 4 µM, Phenomenex, Torrance, CA) under a gradient of mobile phase B (acetonitrile + 0.1% formic acid) in mobile phase A (water + 0.1% formic acid) at a constant flow of 0.6 mL/min over 3 min. The gradient consisted of the following steps: $t = 0$ min (10% B), 0.1 min (10% B), 1.7 min (100% B), 2.2. min (100% B) 2.7 min (10% B). MS-222 and alfaxalone stocks were prepared at 10 mg/mL in sterile water. Sulfamethoxazole stocks were prepared at 10 mg/mL in methanol. A 7-point standard curve of anesthetics in aquaculture water was prepared with both MS-222 and alfaxalone at concentrations between 0.1 and 10 µg/mL. Prior to analysis, study samples were diluted 2- or 100-fold in drug-naive water to ensure that concentrations were below the upper limit of the standard curve. The lower limit of quantitation for study samples was defined as the lowest linear point of the standard curve within each study run. For each standard or study sample, 50 µL of sample was treated with 50 µL of internal standard (0.2 µg/mL sulfamethoxazole in methanol + 0.1% formic acid). Samples were vortexed, centrifuged (10,000 g, 5 min), and supernatant was submitted for liquid chromatography–tandem MS analysis.

Statistical analysis. A power analysis of the primary outcome measure of time to event was conducted using PASS 14 (NCSS, Kaysville UT). A one-way design with 3 groups of sample sizes of 18 each achieves a power of 83% using the Kruskal-Wallis test with a target significance level of 0.050.[9] The null hypothesis is that the SD of the group means is 0.0 and the alternative SD of the group means is 16.5. The average within-group SD assuming the alternative distribution is 35.2. These results are based on 5000 Monte Carlo samples from the null distributions, that is, normal (M1 SD), normal (M1 SD), and normal (M1 SD), and the alternative distributions, that is, normal (M1 SD), normal (M2 SD), and normal (M3 SD). Other parameters used in the simulation were: M1 = 75.0, M2 = 100.0, M3 = 115.0, and SD = 35.0. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC). Assumption of normality and homogeneity of variance were checked using the Shapiro-Wilk normality test and by visual inspection of residual and fitted value plots.[31] A Kruskal-Wallis test was used for overall group comparisons of time to event, maximum and minimum opercular score, maximum and minimum acceleration, and minute-wise distance traveled and mean velocity with post hoc pairwise comparisons made by the Dwass, Steel, Critchlow-Fligner procedure.[15] Descriptive statistics including number of observations, median, lower quartile (Q1), upper quartile (Q3), minimum, and maximum were calculated for these variables. Mixed model analysis of variance using the GLIMMIX procedure with an overdispersion component to the variance function was used for group comparisons of overall distance traveled and mean velocity with post hoc pairwise comparisons made by the Games-Howell procedure. Descriptive statistics including number of observations, mean, SD, minimum, and maximum were calculated for overall distance traveled and mean velocity. Fisher exact tests were used for overall group comparisons of opercular score frequency and frequency of achieving normal swim within 10 min of recovery.[16] Post hoc pairwise comparisons for frequency variables were made by Fisher exact tests with Hochberg adjustment.[17] Mixed model analysis of variance was also used to examine change in anesthetic concentration over time. The level of significance was set at $P \leq 0.05$. All tests were 2-tailed. Missing values were treated as missing-at-random, and values were not imputed.

Results

MS-222 dose optimization pilot. Times are reported as minutes:seconds. Group 1 fish were exposed to 100 mg/L MS-222. Fish 1 reached LOTR at 4:30 post-immersion. The other 2 did not reach this level despite immersion for more than 10 min. Fish 2 lost startle response at 5:42. Fish 3 never lost startle response but did achieve LORR at 3:54 post-immersion. Fish in group 2 were exposed to 150 mg/L MS-222. Fish 2 and 3 reached LOTR at 3:45 and 1:49, respectively. Fish 1 never reached LOTR despite immersion for 6:38. This fish did lose startle response at 1:19 post-immersion. Fish in group 3 were exposed to 200 mg/L MS-222. All fish in this group reached LORR from 0:54 to 0:59, LOSR from 1:06 to 1:08, and LOTR from 1:25 to 1:39. Fish were transferred to the recovery tank approximately 1:00 after LOTR was confirmed. Return of self-righting reflex occurred between 2:00 and 4:08. An MS-222 concentration of 200 mg/L was chosen for the comparison experiment. Statistical analysis was not performed on the pilot study.

Plane of anesthesia. Times are cumulative from point of immersion in the anesthesia tank (that is, the stopwatch did not restart at each timepoint) and are reported in seconds. Assessment of anesthetic depth in zebrafish has been described and was adapted for use in this experiment.[8,36] For this study, the surgical plane of anesthesia (stage III, plane 2) was defined as achieving all of the following: LORR, LOSR, LOTR, and decreased OM. Time to reach surgical plane was shortest for the MS-222 group and longest for the low-dose alfaxalone group.

Overall, fish in the MS-222 group reached a surgical plane of anesthesia more rapidly than did fish in the low-dose ($P < 0.0001$) and high-dose ($P < 0.0001$) alfaxalone groups (Figure 3). Fish in the MS-222 group achieved LORR between 41 and 154 s (median 69.5 s), LOSR between 51 and 165.0 s (median 79.5 s), and LOTR between 70.0 and 211.0 s (median 109.0 s). Fish were moved to the recovery tank between 96.0 and 278.0 s after immersion (median 146.5 s). Fish in the low-dose alfaxalone group achieved LORR between 75.0 and 341.0 s (median, 204.5 s), LOSR between 110.0 and 397.0 s (median 227.5 s), and LOTR between 135.0 and 562.0 s (median 326.5 s). Fish were moved to the recovery tank between 174.0 to 593.0 s after immersion (median 351.5 s). Fish in the high-dose alfaxalone group achieved LORR between 41.0 and 296.0 s (median 119.5 s), LOSR between 78.0 and 350.0 s (median 176.0 s), and LOTR between 126.0 and 473.0 s (median 226.0 s). Fish were moved to the recovery tank between 150.0 and 497.0 s post-immersion (median 257.0 s).

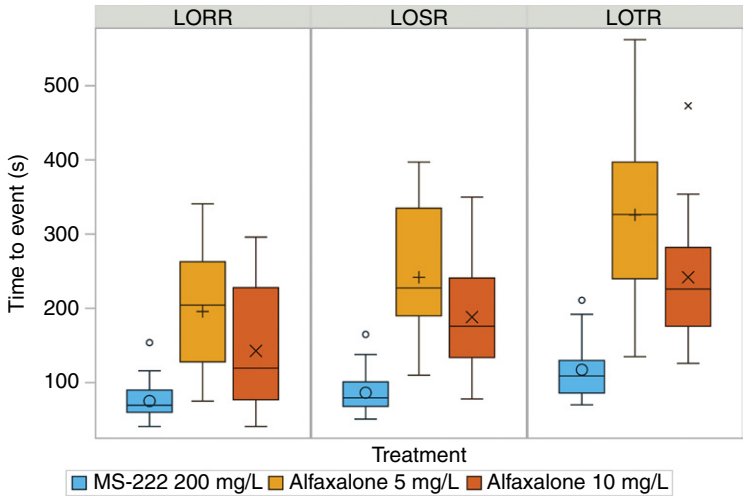

Figure 3. Time in seconds to various anesthetic events. Median for each anesthetic concentration is represented by the following symbols: O = MS-222, + = alfaxalone 5 mg/L, X = alfaxalone 10 mg/L. Boxplot represents the median and the IQR, the marker represents the mean, and the whiskers denote observations falling within a range equal to the first quartile – 1.5 IQR and the third quartile + 1.5 IQR. Small markers outside the whiskers represent extreme observations beyond this range. LORR shows the time to loss of righting reflex for each anesthetic group, which was significantly shorter for the MS-222 group than either alfaxalone group (O = 69.5 s, + = 204.5 s [$P < 0.0001$], X = 119.5 s [$P < 0.0140$]). LOSR shows the time to loss of the startle response, which was significantly shorter in the MS-222 group than either alfaxalone group (O = 79.5 s, + = 227.5 s [$P < 0.0001$], X = 176.0 s [$P < 0.0001$]). LOTR shows the time to loss of tactile response, which was significantly shorter for the MS-222 group than either alfaxalone group (O = 109 s, + = 326.5 s [$P < 0.0001$], X = 226.0s [$P < 0.0001$]).

When compared with fish in the high-dose group, times to LORR, LOSR, and LOTR were all significantly shorter in the MS-222 group ($P = 0.0140$, $P < 0.0001$, and $P < 0.0001$, respectively). When compared with fish in the low-dose group, times to LORR, LOSR, and LOTR were also all significantly shorter in the MS-222 group ($P < 0.0001$ for all). Time moved to recovery tank was statistically significant between the MS-222 group and both the low-dose alfaxalone group ($P < 0.0001$) and the high-dose alfaxalone group ($P = 0.0001$). There were no statistically significant differences for LORR, LOSR, LOTR, or time moved to recovery tank between low-dose and high-dose alfaxalone.

OM was defined by the categories listed in Table 1. Six fish (33.3%) in the MS-222 group reached category 4 (absent). Four (22.2%) reached this level while still in the immersion tank, and 2 (11.1%) were in the recovery tank for 18 s before this was documented. In contrast, OM never went higher than category 2 (shallow) for fish in both alfaxalone groups for the duration of the experiment, with most of each of the alfaxalone groups scoring a category 1 (decreased) (Figure 4). These differences in proportion were significantly different when the MS-222 group was compared with each of the alfaxalone groups ($P < 0.0001$ for both) but not when the 2 alfaxalone groups were compared with each other. Time to maximum OM score (decreased through absent) was significant between the MS-222 and both the low-dose alfaxalone group ($P < 0.0001$) and the high-dose alfaxalone group ($P = 0.0002$), but not between alfaxalone groups.

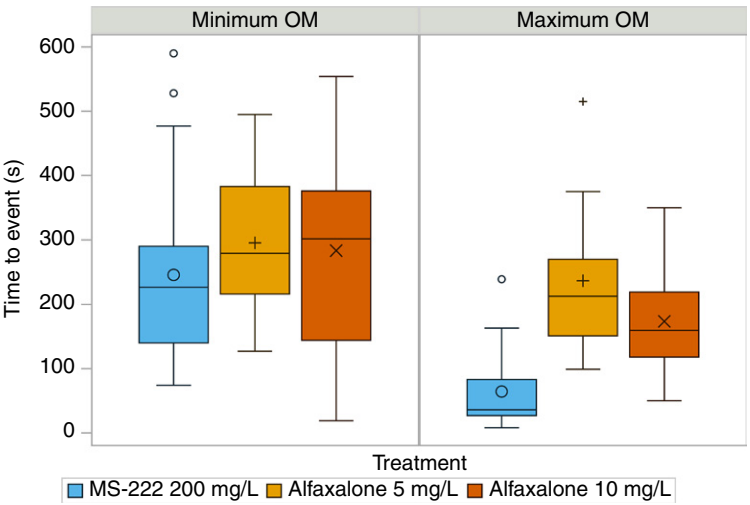

Figure 4. Time in seconds to OM score. Median for each anesthetic concentration is represented by the following symbols: O = MS-222, + = alfaxalone 5 mg/L, X = alfaxalone 10 mg/L. Boxplot represents the median and the IQR, the marker represents the mean, and the whiskers denote observations falling within a range equal to the first quartile – 1.5 IQR and the third quartile + 1.5 IQR. Small markers outside the whiskers represents extreme observations beyond this range. Minimum OM shows the time to return to normal OM score in the recovery tank, which was not significant between any of the groups (O = 226.5 s, + = 279.0 s, X = 301.5 s). Maximum OM shows the time it took each fish to reach their maximum (highest) OM score. Fish in the MS-222 group reached their maximum score (shallow to absent) more quickly than did the fish in either alfaxalone group (decreased to shallow) (O = 36.0 s, + = 212.5 s [$P < 0.0001$], X = 159.5 s [$P = 0.0002$]).

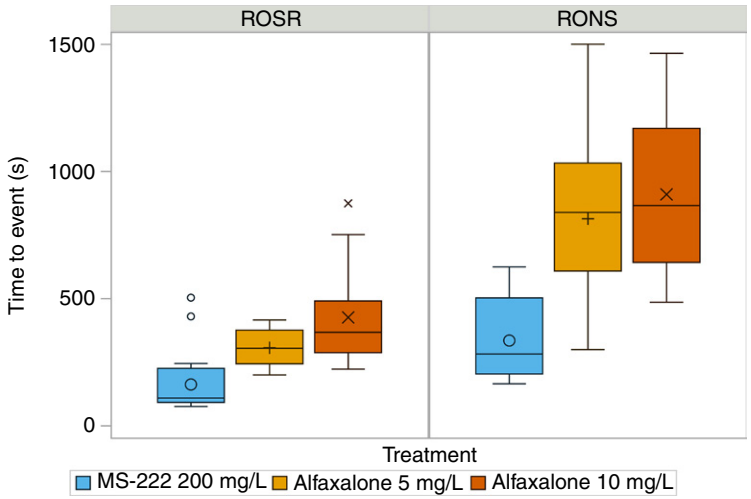

Figure 5. Time in seconds to return to normal after movement to recovery tank. Median for each anesthetic concentration is represented by the following symbols: O = MS-222, + = alfaxalone 5 mg/L, X = alfaxalone 10 mg/L. Boxplot represents the median and the IQR, the marker represents the mean, and the whiskers denote observations falling within a range equal to the first quartile – 1.5 IQR and the third quartile + 1.5 IQR. Small markers outside the whiskers represent extreme observations beyond this range. ROSR shows the time to return of self-righting reflex for each anesthetic group, which was significantly shorter for the MS-222 group than either alfaxalone group (O = 109.0 s, + = 305.0 s [$P = 0.0008$], X = 368.0 s [$P < 0.0001$]). RONS shows the return to normal swim, which was significantly shorter for the MS-222 group than either alfaxalone group (O = 282.5 s, + = 839.5 s [$P < 0.0001$], X = 866.5 s [$P < 0.0001$]).

Recovery. Times are reported in seconds and are cumulative from point of immersion in the recovery tank. Recovery from anesthesia was defined as return of the self-righting reflex (ROSR), return of normal swim (RONS) behavior, and return to normal OM. Fish in the MS-222 group returned to normal swim more quickly than did fish in either of the alfaxalone groups ($P < 0.0001$ for each). There was no significant difference between the alfaxalone groups (Figure 5).

There were significant differences seen in return to self-righting reflex. In the high-dose alfaxalone group, 3 fish (17%) failed to regain the self-righting reflex during the 10-min recovery. In both the low-dose alfaxalone group and the MS-222 group, all fish returned to normal self-righting during the 10-min recovery. Fish in the MS-222 group returned to self-righting reflex more quickly (median 109.0s) than did fish in either the low-dose alfaxalone group (median 305.0 s; $P = 0.0008$) or the high-dose alfaxalone group (median 368.0 s; $P < 0.0001$). There was no significant difference seen in time to the return of self-righting reflex between the alfaxalone groups (Figure 5).

There were significant differences observed in the proportion of fish who returned to normal swim during the 10-min recovery period between the groups ($P < 0.0001$) (Figure 5). In the high-dose alfaxalone group, only 3 fish (17%) returned to normal swim during the 10-min recovery period. In the low-dose alfaxalone group, 4 fish (22%) returned to normal swim during the 10-min recovery period. In contrast, 16 fish (89%) in the MS-222 group returned to normal swim during the 10-min recovery period ($P = 0.0003$ compared with low dose; $P < 0.0001$ compared with high dose). All fish that did not return to normal swim during the 10-min recovery period were moved to a secondary recovery tank until normal swim behavior returned. One fish from the MS-222 group that was moved to the secondary recovery tank returned to normal swim immediately after placement, suggesting normal swim returned within the 10-min recovery period. Due to the prolonged return to normal swim and movement to the secondary tank, times were not exact when fish returned to normal swim, so statistical analysis was not performed on data for this time point.

Although the median time to return to normal OM was lower in the MS-222 group (226.5 s) than in either the low-dose or the high-dose alfaxalone groups (279.0 and 301.5 s, respectively), the differences between groups were not statistically significant.

Differences in minimum acceleration, maximum acceleration, total distance moved, and mean velocity during the entire 10-min recovery period were statistically significant between the groups. The overall minimum acceleration values were lower in the MS-222 (−65.3 to −3.9 cm/s; median −11.8 cm/s) group than in either the low-dose alfaxalone group (−8.1 to −4.2 cm/s; median −6.2 cm/s; $P = 0.0004$) or the high-dose alfaxalone group (−63.8 to −2.9 cm/s; median −6.0 cm/s; $P = 0.0163$). The overall maximum acceleration values were higher in the MS-222 group (3.1 to 64.5 cm/s; median 12.4 cm/s) than in either the low-dose alfaxalone group (3.4 to 10.8 cm/s; median 5.7 cm/s; $P = 0.0003$) or the high-dose alfaxalone group (2.3 to 48.2 cm/s; median 5.7 cm/s; $P = 0.0111$). There were no significant differences between the 2 alfaxalone groups. The MS-222 group showed higher total distance moved (mean 291.4 cm) and mean velocity (mean 0.5 cm/s) compared with the high-dose alfaxalone group (mean 190.2 and 0.3 cm, respectively; $P = 0.0039$ for both). There were no significant differences in total distance moved or mean velocity between the MS-222 group and the low-dose alfaxalone group or between the 2 alfaxalone groups. When looking at these parameters by minute within the recovery period, the minimum and maximum minute acceleration values generally were of greater magnitude in the MS-222 group than those of the 2 alfaxalone groups; however, statistically significant differences were only observed at minute 5 (Figures 6 and 7, respectively). There were no statistically significant differences in minute total distance moved (Figure 8) or mean velocity between groups. There was no statistical significance in weight between any of the groups.

Anesthetic concentration over time. There were no significant changes in anesthetic concentration over time for the MS-222, low-dose, or high-dose alfaxalone tanks. See Table S1 for concentrations over time.

Discussion

In this study, we assessed the efficacy of MS-222 and alfaxalone for achieving a surgical plane of anesthesia by measuring time to loss of righting reflex, loss of startle response by gently

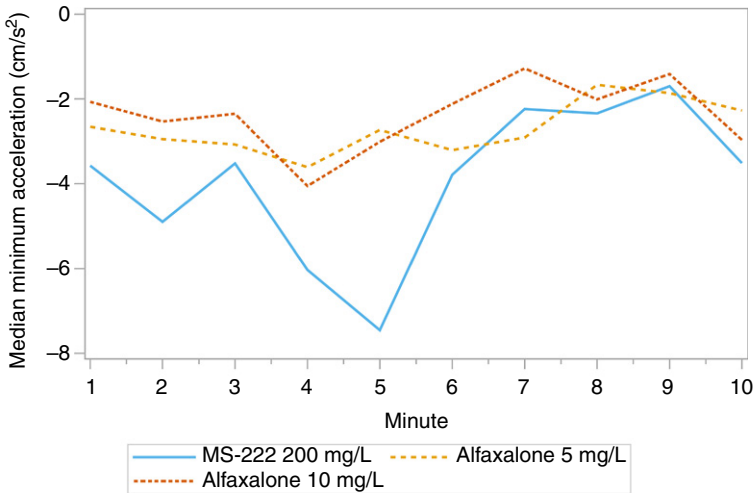

Figure 6. Minimum acceleration per minute during the 10-min recovery. Note the significant drop in acceleration in the MS-222 group at the 5-min mark. This coincides with the median recovery time of 4.75 m and normal bluegill resting behavior. Several bluegill in the alfaxalone groups were not fully recovered at the end of the 10 min.

tapping on the tank, and loss of tactile response by pinching the caudal fin with forceps, similar to those methods reported in other fish species.[8,22,30,36] We have shown that immersion in alfaxalone can be a viable option for providing sedation as well as a surgical plane of anesthesia. Alfaxalone at both concentrations (5 mg/L and 10 mg/L) provided a smooth induction and achieved an adequate and consistent surgical plane, as did MS-222, with no mortality.

All fish reached a surgical plane of anesthesia in this study, although time to do so varied. The time to inducing a surgical plane was shortest in the MS-222 group and longest in the low-dose alfaxalone group (Figure 3). The mean time to induction in the MS-222 group was 109.0 s compared with 226.0 and 326.5 s in the high-dose and low-dose alfaxalone groups, respectively. Return to normal swim was significantly longer in both alfaxalone groups compared with MS-222. These findings are consistent with those reported in other fish species.[5,8,22,27,30,36]

We chose immersion for induction and maintenance in this study. Our laboratory routinely uses this technique for zebrafish with great success. Several have reported on the use of immersion for induction and a water recirculating system for maintenance in other fish species.[27,30] The anesthetic is flushed through the recirculating system and delivered over the gills through the fish's mouth (see Savson and colleagues[30] for an image of the recirculating system in rainbow trout). While this method might be the preferred way to provide consistent anesthetic (and oxygen) delivery over the gills for the entirety of the procedure, the use of a recirculating system would be impractical in smaller fish such as bluegill.

We chose a modification of the manual caudal fin pinch described in other fish species as the noxious stimulus to test LOTR.[22,30] We used forceps to consistently apply the noxious stimulus (Figure 1) as recently reported in zebrafish.[36] The caudal fin is larger in the bluegill when compared with the zebrafish but is smaller than that of rainbow trout and koi. The use of forceps allowed for better visualization of the caudal fin while applying the manual pressure than did the use of fingers.

OM was also considered for induction. We started the comparison experiment with the 200 mg/L MS-222 group and individually maintained the first 4 fish for approximately 1

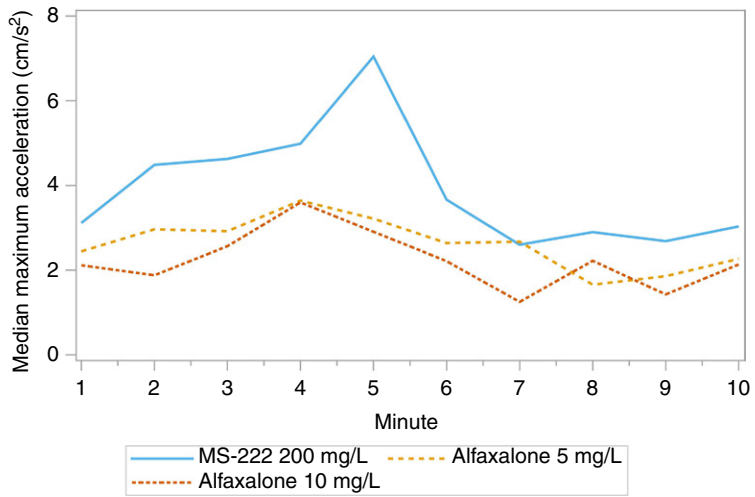

Figure 7. Maximum acceleration per minute during the 10-min recovery. Note the significant acceleration in the MS-222 group slightly before the 5-min mark. This coincides with return to normal swim and a subsequent possible excitatory phase. Acceleration rapidly drops by minute 6 and coincides with normal swim behaviors in bluegill. Several bluegill in each alfaxalone group were not fully recovered at the end of the 10 min. No excitatory phase was noted in these groups during recovery.

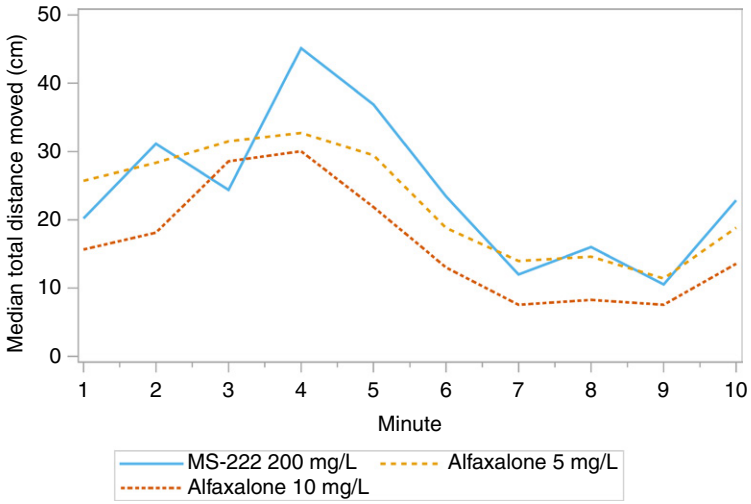

Figure 8. Distance moved per minute during the 10-min recovery. The peak at minute 4 in the MS-222 group correlates with the RONS time and an excitatory phase observed around this time. No excitatory phase was seen in either alfaxalone group. Several fish had not fully recovered by the end of the 10 min.

min after LOTR confirmation. All 4 fish reached an OM score of 4 (absent) during exposure which, lasted from 1 to 5 min. We also noted corneal opacities in all 4 fish during recovery. To prevent this, we adjusted the exposure time to 15 s after LOTR confirmation and did not see this again for the remainder of the experiment. Unbuffered MS-222 has been shown to cause severe corneal damage in fish.[12] However, we buffered this particular MS-222 tank to pH 7.1 prior to immersing any fish. Two other fish exposed to this concentration after shortening the exposure time to approximately 15 s still reached an OM score of 4, and OM returned after 13 or 59 s elapsed. No corneal changes were appreciated in these 2 fish. Our preliminary MS-222 dose determination study consisted of 3 fish per group. The only concentration at which all fish reached what we defined as surgical plane was 200 mg/L. All 3 fish remained in the anesthetic for approximately 1 min after confirming LOTR. All 3 fish reached an OM score of 4 at varying times in the immersion tank. OM returned after approximately 1 to 2 min elapsed. We did not appreciate any corneal changes during recovery in these 3 fish. MS-222 is commonly used as a euthanasia agent at higher doses or prolonged exposure times in many aquatic species.[2] Death occurs due to decreased nervous and cardiovascular function.[2] We do not think an acidic environment caused these corneal changes and suspect it was due to possible hypoxia associated with prolonged cessation of OM. It is also possible that our chosen MS-222 concentration of 200 mg/L could be a euthanasia concentration for this species when exposed for longer times and could explain why the 4 fish developed corneal opacities, although more work is needed to determine this.

Interestingly, OM never reached rare (score of 3) or absent (score of 4) in any fish in either the low-dose or high-dose alfaxalone group in this study (Figure 4). Respiratory depression is a commonly reported side effect for alfaxalone regardless of route of administration. Prolonged apnea was reported in 3 of 6 koi after a 10 mg/kg alfaxalone intramuscular injection with a 33% mortality rate.[3] Respiratory depression has also been reported in other fish species.[5,22,27,36] One study reported cessation of OM in 22 out of 22 black spot bards and 18 out of 22 peacock cichlids when in 5 mg/L alfaxalone for more than 30 s.[41] An initial excitatory phase followed by OM cessation was observed in goldfish exposed to 6, 7, and 9 mg/L alfaxalone.[22] While a decreased or shallow OM is considered appropriate for a surgical plane of anesthesia, it is possible that the fish in this study were not maintained long enough to appreciate this.

Several fish in both alfaxalone groups did not respond to the tactile stimulation test and were considered at a surgical plane based on our definition but flinched in the net when transferred from immersion to recovery. When in the recovery tank, fish still appeared anesthetized (LORR), although depth could not be assessed due to the EthoVision XT camera tracking fish movement during recovery. At this study's conclusion, the observer reviewed footage of every anesthetic event and noticed in several fish in both alfaxalone groups that OM would either slow or cease during the tactile response test but would return to the score prior to application as soon as the stimulus was removed. This was not observed in real time as the observer was focusing on assessing tactile response. These findings suggest these fish might not have been fully anesthetized for surgical procedures despite LOTR confirmation. A study in koi found that 4 of 6 fish reacted to an intramuscular injection into the epaxial muscles at 1 mg/L alfaxalone.[27] Longer exposure times may be required to achieve appropriate depth for invasive procedures when using alfaxalone in bluegill. The authors concur with previous recommendations that anesthetic protocols should be tested on a few fish prior to manipulation to ensure that appropriate depth is achieved for the procedure.[30,40] More research is needed to develop the phases of anesthesia and an anesthetic depth scale for bluegill. Further study is also needed to determine analgesic effects of both anesthetics for painful procedures (fin clipping, tagging, sample collection) in this species.

EthoVision XT software, which tracks movement and acceleration over time, was used to document the recovery period for all 3 groups. Under normal conditions, bluegill fish are sedentary, generally swimming only to feed, avoid predators, or defend nesting sites. In this study, total distance moved, mean velocity, and minimum and maximum acceleration over the entire 10-min recovery period were statistically significant when each alfaxalone group was compared with MS-222. The median time to recovery for MS-222 was 282.5 s, but was considerably longer in both alfaxalone groups: 681.0 s in the low-dose group and 673.5 s in the high-dose group (Figure 5). At 5 min, the median maximum acceleration for the fish in the MS-222 group was 7.0 cm/s (P = 0.0171). This acceleration coincides

with an excitatory phase in recovery seen only in this group. When righting reflex returned, several fish in this group swam and thrashed for several seconds before settling back to normal bluegill swim behavior (Figure 7). This was not appreciated in either alfaxalone group.

Adverse events, other than respiratory depression, were few. Only one fish in the entire study regurgitated in the anesthetic tank and none regurgitated in the recovery tank. This fish was inadvertently fed within the defined fasting period. No deaths occurred in any group during this procedure.

We theorized that repeated metabolism of the anesthetic would decrease the available concentration in the tank over time. To help alleviate this, all solutions were prepared immediately before each session. Anesthetic concentrations were not tested twice in the same day. For example, the first solution tested on day one was MS-222, and the second solution was the low-dose alfaxalone group. Nine fish were anesthetized at each concentration before the solution was changed. The longest any solution was used was approximately 3 h. Our data suggest that anesthetic concentration remained consistent over the course of the experiment, further supporting alfaxalone as an alternative to MS-222 for anesthesia in clinical and research (that is, field) conditions.

Several limitations may exist when choosing either anesthetic for general anesthesia in bluegill. Our study found that recovery was prolonged in both alfaxalone groups when compared with MS-222. Dose-dependent recovery for alfaxalone was reported in koi where fish maintained at 1 mg/L alfaxalone recovered in roughly half the time as those maintained at 2.5 mg/L.[27] In contrast, we did not appreciate any differences in recovery times for either alfaxalone group. In fact, 2 fish in the low-dose group took at least 30 min to fully recover, although the authors admit these fish were not as closely observed while in the secondary recovery tank. We used continuous immersion instead of a recirculating system so our concentrations of alfaxalone were considerably higher. When using a recirculating system, fish are induced in an immersion tank, then maintained at a lower concentration on the recirculating system. Here, the anesthetic flows over the gills continuously, which requires lower anesthetic concentrations.[10,27,30] It is possible that differences in recovery time could be seen at a lower concentration when compared with our higher concentrations; however, induction times could be longer. Prolonged induction or recovery could be a major hinderance in selecting a superior anesthetic in certain field conditions.

Another considerable limitation is that alfaxalone is a controlled substance in the United States.[33] Anyone interested in using this agent must meet multiple requirements to order and prescribe controlled substances, which include registering with the Drug Enforcement Administration and keeping detailed records of use and waste. Traveling with controlled substances and maintaining records while in field conditions can present additional challenges. On the other hand, MS-222 is readily available to anyone without the need to register with the Drug Enforcement Administration.

A major limitation with MS-222 is the 21-d withdrawal period that must be met before fish can enter the food supply. Steps must be taken to ensure the fish will not enter the food chain within those 21 d, but resources can be lacking to meet this requirement.[30] A recent study found that alfaxalone clears rainbow trout tissues within 72 h post-administration. Although not currently approved for use in food animals, withdrawal periods could be drastically reduced with alfaxalone use.[30] Further research is needed to determine whether short clearance times are similar for other food and food producing species, including bluegill.

The observer noted that induction appeared much smoother in both alfaxalone groups. The fish did not react as vigorously when placed in these anesthetic tanks compared with the MS-222 fish. These fish would swim and thrash, sometimes violently, when first placed in the anesthesia tank and the skin would darken almost immediately, suggesting the animal was immediately stressed when placed in the MS-222 solution despite appropriate buffering. Although the skin darkened at both alfaxalone concentrations, anecdotally it seemed to occur later in the induction phase and was not as drastic (dark) when compared with the MS-222 fish. Fish also appeared more docile when placed in either alfaxalone concentration compared with MS-222 fish in contrast to what was seen in goldfish exposed to 7 and 9 mg/L alfaxalone.[22] A 2-min excitatory phase was observed when first placed in the immersion tank. We did not appreciate this excitatory phase during alfaxalone induction in the current study. When fish were losing righting reflex in the alfaxalone groups, they would gently roll from side to side until finally coming to rest upside down on the bottom of the tank. Recovery appeared smoother for the alfaxalone groups as well. The observer noticed an excitatory phase in the MS-222 recovery tank. These fish would dart and thrash for several seconds immediately after righting reflex returned before calming down and returning to normal swim. This was not observed in either alfaxalone group. These behaviors were not expected, and the experimental design did not include methods to capture this behavior. Further studies could compare the different behavioral responses experienced between the 2 agents to further elucidate this phenomenon.

Overall, we demonstrated that alfaxalone administered via immersion is a viable option for providing rapid and reliable sedation and anesthesia in bluegill fish and the response is dose-dependent. From the observer's perspective, alfaxalone provided a smooth induction and recovery compared with MS-222, which could be invaluable for mitigating compassion fatigue.

Supplementary Materials

Table S1. Concentration of MS-222 or alfaxalone in the anesthetic tank over time

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Disclaimers

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Conflict of Interest

The authors have no conflicts of interest to declare.

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