2011

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*Articles*

# Spearmint (*l*-Carvone) Oil and Wintergreen (Methyl Salicylate) Oil Emulsion Is an Effective Immersion Anesthetic of Fishes

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## Abstract

This study evaluates the effects of a spearmint (*l*-carvone) and wintergreen oil (methyl salicylate) emulsion (CMSE) on age 1 landlocked Atlantic salmon *Salmo salar sebago* (hereafter salmon). Salmon were immersed in either 257 µl/L CMSE or 75 mg/L tricaine methanesulfonate (MS-222) to induce anesthesia (stage 4), useful for emersion and noninvasive husbandry procedures, and then salmon were recovered in fresh water. Induction was quicker in the CMSE group; however, recovery was quicker in the MS-222 group. A second experiment was conducted in which salmon were immersed in 257 µl/L CMSE for 8.5 min, or 75 mg/L MS-222 for 8.5 min in order to compare electrocardiographs during deeper anesthesia (stage 5) between salmon continuously immersed in CMSE to those continuously immersed in MS-222. Because salmon remained sedated longer after CMSE exposure than after MS-222 exposure, a third group of salmon was immersed in 257 µl/L CMSE for just 2.5 min before undergoing the 6-min electrocardiograph procedure. Anesthesia induction rates, recovery rates, and electrocardiographs of salmon anesthetized with CMSE were comparable to salmon anesthetized with MS-222. Salmon anesthetized with CMSE and then transferred immediately to fresh water had more stable heart rates than salmon anesthetized with either MS-222 or CMSE continuously. Salmon bathed continuously in CMSE showed clinical signs of increasing anesthetic depth including decreasing heart rate, decreasing respiration rate and electrocardiograph abnormalities. The CMSE, with its mint and wintergreen concentrations less than in household products such as chewing gum, toothpaste, and mouthwash, is a potent, rapid-acting immersion fish anesthetic comparable to MS-222 for stages 4 and 5 anesthesia.

Keywords: anesthesia; electrocardiography; food safety; aquaculture

Received: March 31, 2011; Accepted: September 1, 2011; Published Online Early: September 2011; Published: December 2011

Citation: Danner GR, Muto KW, Zieba AM, Stillman CM, Seggio JA, Ahmad ST. 2011. Spearmint (*l*-carvone) oil and wintergreen (methyl salicylate) oil emulsion is an effective immersion anesthetic of fishes. *Journal of Fish and Wildlife Management* 2(2):146–155; e1944-687X. doi: 10.3996/032011-JFWM-025



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## Introduction

In the United States, no approved fish anesthetic exists with a 0-d preslaughter drug (allowed to be consumed by humans on the same day as application of drug) withdrawal period, nor is there a 0-d preangling fish anesthetic approved. The U.S. Food and Drug Administration (FDA)-approved and commonly used fish anesthetic tricaine methanesulfonate (MS-222; Argent Chemical Co., Redmond, WA), has a 21-d preslaughter drug withdrawal period. Consequently, food fish cannot be anesthetized within 21 d of slaughter, nor can wild fish

be anesthetized and released back into public waters when they might be recaptured and consumed by anglers during the 21-d drug withdrawal period. An efficacious fish anesthetic that is safe for 0 d before slaughter and immediate release after use has been the goal of state, federal, and private fisheries professionals for decades (Marking and Meyer 1985; Schnick 2005, 2006). The Aquaculture Association Drug Approval Partnership (http://www.fws.gov/fisheries/aadap/home.htm) was formed as an alliance of public and private fisheries and aquaculture professionals that would target their limited resources toward more effective aquaculture drug approval in the United States. A top priority of the Aquaculture Association Drug Approval Partnership is to find a 0-d preslaughter withdrawal period fish anesthetic.

Anesthesia is a triad of narcosis, analgesia, and skeletal muscle relaxation (Brown 1993). A multifarious assortment of off-label drugs and unapproved substances will anesthetize fishes (Brown 1993; Neiffer 2007). However, drug residue levels, carcinogenicity, metabolite toxicity, or the absence of this type of pharmacological information has prevented any drug from being FDA approved for 0-d preslaughter withdrawal. Routes and methods of delivering a chemical fish anesthetic include immersion; parenteral, intramuscular, or intraperitoneal injection; and oral ingestion. Myriad routes and methods of drug delivery complicate ichthyio-pharmacology and further stifle FDA approval. Of the anesthetic delivery methods, immersion is the most common and most practical means of anesthetizing batches of fish, or fishes, and is routine procedure in aquaculture and resource fisheries management (Wedemeyer 2001). Determining drug residue levels and other pharmacological information on immersion anesthetics delivered to different fish species, different fish sizes, and different exposure durations has proven too costly to drug manufacturers for a "Minor Use, Minor Species" market that is small; the Minor Use and Minor Species Animal Health Act of 2004, commonly referred to as the "MUMS Act," was signed into law on August 2, 2004 (USFDA 2011). The law is intended to make more medications legally available to veterinarians and animal owners to treat minor animal species, defined as animals other than cattle, horses, swine, chickens, turkeys, dogs, and cats; and uncommon diseases in the major animal species (USFDA 2011). Consequently, the Aquaculture Association Drug Approval Partnership has stepped in to coordinate Investigational New Animal Drug and other FDA-approval steps with manufacturers of potential 0-d preslaughter withdrawal fish anesthetic compounds. One of these potential fish anesthetic compounds is an emulsified mixture of two common human food substances: spearmint oil (*l*-carvone) and wintergreen oil (methyl salicylate).

Spearmint *Mentha spicata* has been consumed by humans for millennia; it has been written into mythical and religious tales, and today mint is the third most popular flavor in the world behind vanilla *Vanilla planifolia* and citrus (Hayes et al. 2007). In antiquity, spearmint was known as the herb of hospitality (de Carvalho and Da Fonseca 2006). Today, the world market for spearmint oil is approximately 1,500 tons/y (Peterson 2004). It is used in cuisine, candy, chewing gum, cosmetics, toothpaste, tobacco products, and pharmaceutical preparations (Chen et al. 2010).

Carvone is the component of the spearmint oil that gives it a minty fragrance and flavor. Caraway *Carum carvi* seeds, dill weed *Anethum graveolens*, mandarin orange *Citrus reticulata*, peels, peppermint *Mentha piperita*, and many fruits contain mixtures of both enantiomers of carvone and related substances that give them their unique fragrances. *l*-Carvone can be biosynthesized from tangerine peels or extracted from spearmint plants (de Carvalho and Da Fonseca 2006). In the United States, *l*-carvone is available from both synthetic and natural sources (Lawrence 2007). Pharmacologically, *l*-carvone is reported to be a sodium channel blocker (de Sousa et al. 2007; Goncalves et al. 2008). *l*-Carvone's anesthetic effect on the peripheral and central nervous systems include central nervous system depression, antinociceptive effects, sedation, and anticonvulsant-like activity (de Sousa et al. 2007; Goncalves et al. 2008; Oliveira et al. 2008).

Wintergreen oil is a natural product of many plants and is considered to be an antiherbivore defense compound. Wintergreen oil is used as a rubefacient in human and veterinary liniments and as a flavoring agent in cuisine, chewing gum, candy, toothpaste, tobacco products, and pharmaceutical preparations (USEPA 2005; Chen et al. 2010). Methyl salicylate is the component of wintergreen oil that gives it a cool and minty fragrance and flavor. Methyl salicylate can be synthesized by esterifying salicylic acid with methanol, but for centuries it was distilled from twigs of sweet birch *Betula lenta* and eastern teaberry *Gaultheria procumbens*. In the United States, methyl salicylate is available from both synthetic and natural sources. It is available Kosher, Halal, and GMP. Methyl salicylate is purported to be a nonsteroidal anti-inflammatory drug with pharmacological effects comparable to salicylic acid (Stevens and Warren 1964). Salicylate's pharmacological properties include antinociceptive effects, anti-inflammatory effects, inhibition of platelet aggregation, and toxicity (Davison et al. 1961; Ichiyama et al. 2002; Soo Hoo et al. 2003; Calvo 2006).

Although both *l*-carvone and methyl salicylate are capable of anesthetizing fishes individually (data not shown), the mixture of *l*-carvone and methyl salicylate is a superior anesthetic with lower doses, quicker induction times, quicker recovery times, and less muscle movement in emersed fishes. The synergistic effects of anesthetic drug combinations are well documented and form the basis of medical plans for pain management (Skarda et al. 1995; West et al. 2007). The synergistic combination of a nonsteroidal anti-inflammatory drug and $Na^+$ channel blocker are particularly adept at analgesia of the peripheral nervous system and well suited for topical application (Liedtke 2006). Based on the modulated receptor hypothesis, the combination of a nonsteroidal anti-inflammatory drug and a $Na^+$ channel blocker enhances anesthesia by increasing the affinity of $Na^+$ channel blockers with high lipid solubility to inactivated state $Na^+$ channels (Hiji et al. 1987; Ichikawa

1987). Salicylate enhances tonic and phasic blocking of $Na^+$ channels induced by class 1 highly liposoluble antiarrhythmic agents (Tanaka et al. 1999). The spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion (CMSE) label reveals a 7:1 ratio of *l*-carvone to methyl salicylate, and it is applied topically to fish by immersion. The emulsion takes advantage of both the synergistic effects of a nonsteroidal anti-inflammatory drug paired with a $Na^+$ channel blocker and their aptitude for peripheral nerve analgesia when applied topically.

This study determined the induction rate, recovery rate, and electrocardiograph pattern of fish anesthetized with a novel *l*-carvone– and methyl salicylate-based anesthetic and compared them with the commonly used FDA-approved fish anesthetic MS-222 (*Supplemental Material*, Table S1; http://dx.doi.org/10.3996/032011-JFWM-025.S1). We report that CMSE is an effective nonnarcotic, norbarbiturate immersion fish anesthetic comparable to MS-222 for stages 4 and 5 anesthesia.

## Methods

### Immersion anesthetic formulation and preparation

The CMSE (Maine Conservation Medicine Center, Inc., Waterville, ME) is composed of 28.4% *l*-carvone (*p*-mentha-6,8-dien-2-one), 4% methyl salicylate (methyl-2-hydroxybenzoate), 25% glycerin, and 5% polysorbate 80 in an aqueous solution. The ingredients are blended aggressively until a brilliant white 5-μm micelle emulsion forms. The liquid anesthetic is dispensed by volume with 1 mL of CMSE containing 75 μl/L *l*-carvone, 11 μl/L methyl salicylate, and 300 μl/L emulsifiers (i.e., 86 μl/L of the combination and 300 μl/L emulsifiers). The stock solution has a pH of 5.8. The manufacturer's working draft FDA drug label was followed with an empirical dose of 257 μl/L CMSE used to anesthetize landlocked Atlantic salmon *Salmo salar Sebago* (hereafter, salmon). The pH of the anesthetic bath remained at 6.8 after the 30 mL of CMSE was added to the 38 L of hatchery water in an anesthetic tank. The immersion anesthetic solution was not buffered because adjusting the pH of the immersion solution is not part of the draft label directions for CMSE. The effect of CMSE on the hatchery water's pH best fit a line pH = 0.0107 μl + 6.8 at 12°C.

The MS-222 powder (Argent Chemical Co.) was weighed on a gram scale. The 75 mg/L dose determined for the 38-L anesthetic tank was 2.8 g of MS-222. The pH of the anesthetic bath decreased from 6.8 to 3.7 after the MS-222 was added. The immersion anesthetic solution was not buffered because adjusting the pH of the anesthetic solution is not part of the FDA label directions for MS-222's use (Welker et al. 2007). The hatchery-specific effect of MS-222 on the water's pH was measured with MS-222 concentrations between 1,000 and 16 mg/L using a pH meter. The effect of MS-222 on the hatchery water's pH best fit a line pH = 0.1179 mg + 3.1 at 12°C.

### Subjects

We obtained 130 age 1 salmon (mean body mass ± SD, 117 ± 26 g; mean total body length, 222 ± 14 mm) from the Maine Department of Inland Fisheries and Wildlife, Ela Fish Rearing Station, Embden, Maine. Salmon came from a larger lot previously inspected for, and found negative of, fish pathogens of regulatory concern (Wiggins 2001; AFS-FHS 2004).

### Stage 4 anesthesia experiment

Three replicates of 10 salmon each were netted out of a 40,000-L circular rearing tank, placed directly into a 38-L tank of water (pH 6.8 at 12°C with 86% oxygen saturation, <10 mg/L salt), and pretreated with 257 μl/L CMSE. Another three replicates of 10 salmon were anesthetized with 75 mg/L MS-222 at the same initial water temperature, pH, oxygen saturation, and salinity. Salmon were observed for their reaction to the anesthetic solution, and the time to stage 4 anesthesia was recorded for the first and last fish in each replicate. Salmon were considered handleable when they reached stage 4 anesthesia (i.e., loss of equilibrium, no effort to right itself, decreased muscle tone, some reaction to strong stimuli; Brown 1993). Fish were weighed, measured, and transferred into 38 L of fresh untreated water for recovery. Because the 10 fish in each replicate did not induce and recover synchronously, the time to recovery was recorded for the first and last fish in each replicate. Salmon were considered recovered when they returned to normal (i.e., righted itself, swim actively, muscle tone normal; Brown 1993). A mean time to stage 4 anesthesia and a mean time to recovery were calculated between the first and last fish in each replicate. Salmon were monitored for survival for 14 d.

### Stage 5 anesthesia experiment

Sixty salmon were randomly assigned to one of three treatment groups to compare the effects of either brief CMSE exposure (CMSE-FW) or continuous CMSE exposure (CMSE-CE) on a salmon's electrocardiograph (ECG) and the effects of continuous MS-222 exposure (MS-222-CE) on a salmon's ECG. Salmon were anesthetized in the 38-L tanks as described under Stage 4 anesthesia experiment. The time to light anesthesia (stage 5, Table 1) was recorded and standardized to 2.5 min for all three treatment groups based on our previous experience with CMSE and MS-222 (Brown 1993). Label directions for MS-222 state that fish may be left in the anesthetic solution during procedures for up to 14 min. There are no directions for euthanasia of fishes with MS-222; this is an off-label use of MS-222 (Smit and Hattingh 1979; MacAvoy and Zaepfel 1997).

Salmon in the first treatment group ($n$ = 20), CMSE-FW, were immersed in 257 μl/L CMSE for 2.5 min and then immediately transferred to fresh water for the ECG procedure. Salmon in the second treatment group ($n$ = 20), CMSE-CE, were immersed in 257 μl/L CMSE continuously for the duration of the ECG procedure. Salmon in the third treatment group ($n$ = 20), MS-222-CE, were immersed in 75 mg/L MS-222 continuously for the duration of the ECG procedure. To control for treatment effects of the invasive procedure, salmon in each treatment group were anesthetized in pairs. One salmon in each pair underwent the ECG procedure, and

**Table 1.** Anesthetic stages, descriptions, and fish behaviors associated with each anesthetic stage. Table modified from Cotter and Rodnick (2006).

| Stage | Descriptor | | Fish behavior |
|---|---|---|---|
| 0 | Unanesthetized and recovered | Normal | Reactive to stimuli; opercular rate appropriate for species, water temperature and oxygen saturation, muscle tone firm; equilibrium normal. |
| 1 | Light sedation | Handleable (noninvasive procedures) | Slight loss of reactivity to tactile stimuli; opercular rate decreased slightly; equilibrium normal. |
| 2 | Deep sedation | | Total loss of reactivity to weak stimuli, reactive to only very strong stimuli; opercular rate decreased or increased slightly; equilibrium normal. |
| 3 | Partial loss of equilibrium | | Reactive only to very strong stimuli; increased or decreased opercular rate; partial loss of muscle tone; erratic swimming and disequilibrium. |
| 4 | Loss of equilibrium | Surgical (invasive procedures) | Slow, regular opercular movements; total loss of equilibrium and muscle tone. |
| 5 | Loss of reflex reactivity | | Total loss of reactivity to tail pinch; opercular movements slow; heart rate slow. |
| 6 | Medullary collapse | | Opercular movements stop, usually followed by cardiac arrhythmia or arrest. |
| D | Death | | Apnea; no muscle tone; asystole. |

the other control cohort was anesthetized and recovered but did not undergo the invasive procedure.

## ECG procedure

The ECG procedure began without delay after the salmon were immersed in the anesthetic for 2.5 min. Salmon were netted out of the anesthetic tank and placed in dorsal recumbency on a wet sponge sling partially submerged in either fresh water (CMSE-FW) or anesthetic solution (CMSE-CE and MS-222-CE). Each salmon was positioned at a slightly negative incline so that the head and gills would remain submerged but the ECG electrodes, belly, and tail would not be submerged (Figure 1). Two 12-mm, 29-gauge needle electrodes (AD Instruments, Inc., Colorado Springs, CO) were positioned in the muscle tissue around the pericardial cavity to record the heart's electrical activity; a third grounding electrode was placed in the hypaxial muscles of the caudal belly area. The electrodes were positioned as described by Cotter and Rodnick (2006, 2007). The needle electrodes were connected to an ECG amplifier (PowerLab/4ST Bio Amp; AD Instruments, Inc.), computer (Imac 4.1; Apple, Inc., Cupertino, CA), operating system (OS X, version 10.4.11; Apple, Inc.), and ECG software (Chart software, version 5.5; AD Instruments, Inc.). A separate push button switch was connected to the ECG amplifier to record respiratory effort. No attempt to artificially irrigate the gills beyond keeping them submerged was allowed so that autogenous buccal pumping efforts could be recorded. The ECG and respiration rate were recorded for 6 min, after which the electrodes were removed and the salmon were transferred into a fresh water recovery tank (pH 6.8 at 12°C, 38 L with 86% oxygen saturation). Recovery time was recorded. Control cohorts from each pairing were removed from the treatment tank and placed in fresh water to begin their recovery period at the same instant as the electrocardiographed salmon. Fish were monitored for 14 d after the procedure for signs of postprocedural morbidity or mortality. Separate sponges were used for CMSE-FW, CMSE-CE and MS-222-CE treatment groups to prevent cross-contamination from the different anesthetics.

## Statistics

Data were tested for normality using the Shapiro–Wilk test and normality plots. Means, standard deviations, and standard errors were calculated for normal data distributions. Nonparametric tests were chosen for nonnormal data. Levene's test was used to test the assumption of equal variances between groups. Values reported to determine differences amongst groups were calculated using statistical Analyze It software (Analyze It, Inc., Leeds, England, United Kingdom) and Systat 12 software (Systat Software, Inc., Chicago, IL). Values for heartbeat segments such as QRS duration, QRS amplitude, and QT duration data for ECG parameters and respiration rates were measured directly from individual ECG files within the ECG software using cardiac parameters described by Cotter and Rodnick (2006, 2007). A 20-s interval was selected from each minute of the 6-min ECG procedure to measure the heart rate. Three individual QRST complexes were measured for QRS amplitude, QRS duration, and QT duration from each corresponding 20-s interval of the matching minute (*Supplemental Material*, Figure S1; http://dx.doi.org/10.3996/032011-JFWM-025.S1).

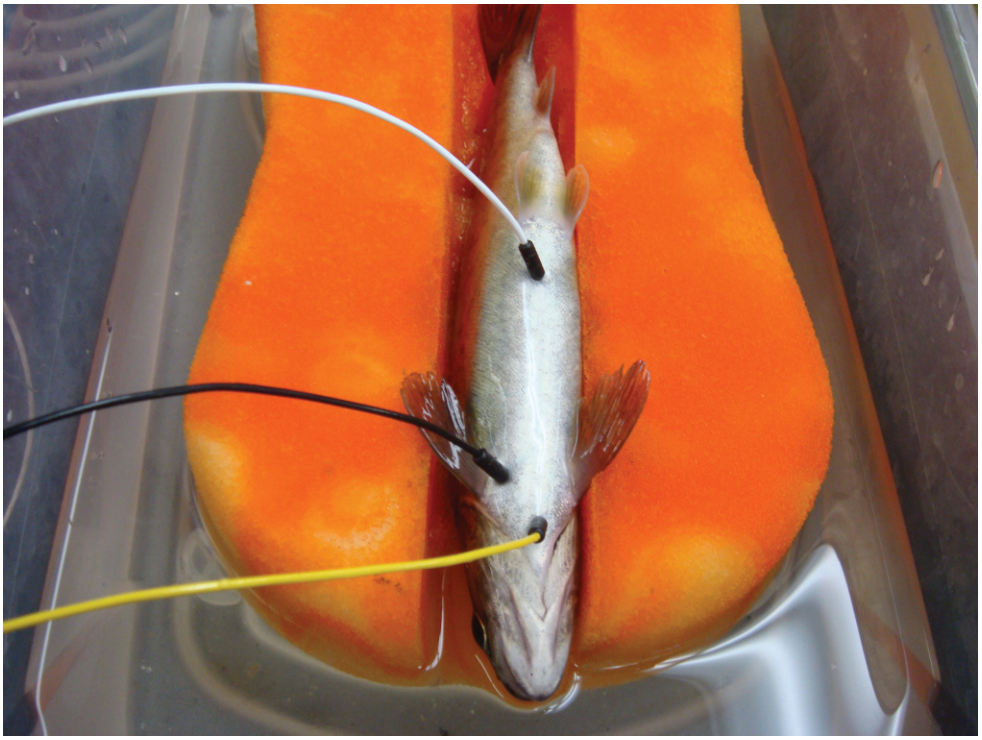

**Figure 1.** Landlocked Atlantic salmon *Salmo salar sebago* being positioned for electrocardiograph procedure. Needle electrodes were placed in muscle tissue surrounding pericardial cavity. Salmon remained partially submerged in water to keep gills moist; no additional gill irrigation was provided. Salmon were restrained only by anesthetic and held in dorsal recumbency position by a trough cut into the sponge.

## Results

Salmon anesthetized with CMSE displayed more excitability before induction than MS-222–anesthetized salmon. These salmon displayed protective behaviors, such as flaring opercula, coughing, and anxious swimming and splashing, when immersed in the CMSE anesthetic bath. The anxious behavior was short-lived but nevertheless a noteworthy observational difference between CMSE and MS-222 anesthetic induction.

Salmon anesthetized in CMSE reached stage 4 anesthesia in as quickly as 54 s; the time (mean $\pm$ SE) to this anesthetic level for the three replicate groups was 1.2 $\pm$ 0.1 min (*Supplemental Material*, Table S2; http://dx.doi.org/10.3996/032011-JFWM-025.S1). Salmon anesthetized in MS-222 reached stage 4 anesthesia as quickly as 1.1 min; the time to this anesthetic level for the three replicate groups was 1.7 $\pm$ 0.2 min (Figure 2). Therefore, CMSE had a slightly quicker induction time to stage 4 anesthesia than MS-222. The difference in mean time to stage 4 anesthesia between the CMSE and MS-222 was statistically significant ($t = 5.1$, df = 8, $P < 0.01$). Stage 4 anesthesia was adequate for noninvasive routine aquaculture and fisheries procedures (e.g., grading, sorting, spawning, and measuring total body mass and total body length). The difference in induction time for stage 4 anesthesia between CMSE and MS-222 is not due to differences in fish size. Total body mass and length were normally distributed. Student's independent *t*-tests showed no statistical difference in either total body mass ($t = 1.67$, df = 58, $P = 0.10$) or length ($t = 0.32$, df = 58, $P = 0.75$) between salmon anesthetized with CMSE or MS-222.

Salmon recovered from CMSE anesthesia as fast as 1.5 min after transfer from CMSE to fresh water, and the mean recovery time for the three replicate groups was 2.5 $\pm$ 0.4 min. The longest recovery time for any CMSE-anesthetized salmon was 3.6 min. Salmon recovered to an upright position within 1.1 min after transfer from MS-222 to fresh water, and the mean recovery time for the three replicate groups was 2.1 $\pm$ 0.3 min (Figure 2). Therefore, CMSE had a slower recovery time from stage 4 anesthesia than MS-222. The difference in mean recovery time from stage 4 anesthesia between the CMSE and MS-222 was statistically significant ($t = 3.62$, df = 8, $P < 0.01$). These findings demonstrate that CMSE has comparable induction and recovery times (stage 4 anesthesia) to MS-222.

For salmon anesthetized at stage 5, survival rate was comparable among all treatments, and there was no statistical difference in survival between any of the six cohorts (Pearson's $\chi^2 = 0.04$, df = 2, $P = 0.98$; *Supplemental Material*, Table S3; http://dx.doi.org/10.3996/032011-JFWM-025.S1). The CMSE-FW treatment group had 100% survival in both the anesthesia-only cohort and the anesthesia-ECG cohort. The CMSE-CE treatment group had 70% survival in the anesthesia-only cohort and 70% survival in the anesthesia-ECG cohort. The MS-222-CE treatment group had 90% survival in the anesthesia-only cohort and 80% survival in the anesthesia-ECG cohort.

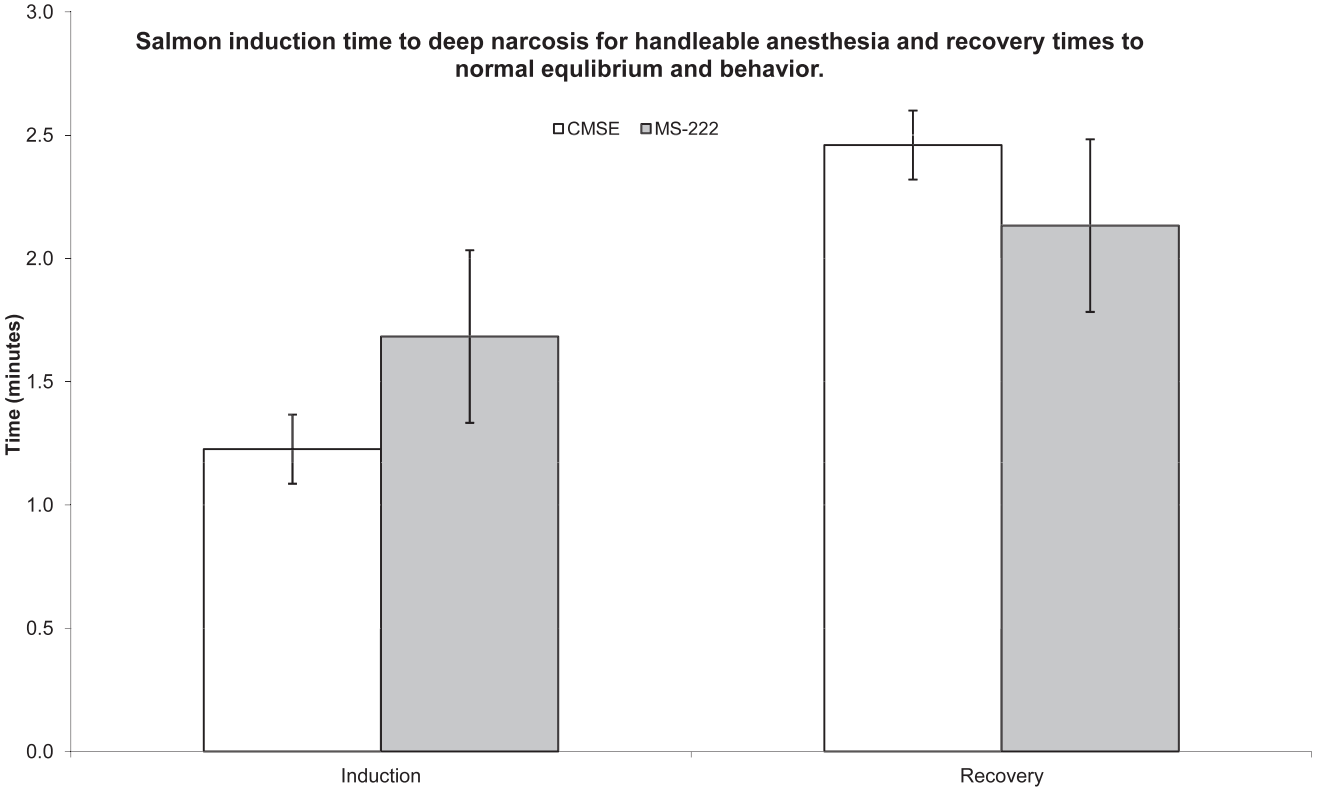


**Figure 2.** Anesthesia induction and recovery times for landlocked Atlantic salmon *Salmo salar sebago* anesthetized to stage 4 anesthesia (i.e., loss of equilibrium, no effort to right itself, decreased muscle tone, some reaction to strong stimuli; Brown 1993) with spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion fish anesthetic or tricaine methanesulfonate fish anesthetic. Salmon were considered handleable when they reached stage 4 anesthesia.

Recovery times for salmon recovering from stage 5 anesthesia were not significantly longer than salmon recovering from stage 4 anesthesia. The mean recovery times for anesthesia-only cohorts from CMSE-FW, CMSE-CE, and MS-222-CE were 4.3 ± 0.5, 7.5 ± 0.5, and 3.4 ± 0.5 min, respectively.

The heart rates in the treatment groups were comparable and not significantly different (Levene's test: $F_{2,27} = 0.26$, $P = 0.77$; *Supplemental Material*, Table S4; http://dx.doi.org/10.3996/032011-JFWM-025.S1). The mean heart rates for the ECG procedure were CMSE-FW = 38 ± 2 beats/min, CMSE-CE = 30 ± 2 beats/min, and MS-222-CE = 33 ± 2 beats/min (Figure 3; Table 2). Notwithstanding, the heart rates of CMSE-CE and MS-222-CE significantly decreased with time, whereas the heart rate of CMSE-FW remained stable (Kruskal–Wallis test: CMSE-CE = 17.6, df = 5, $P = 0.0035$; MS-222-CE = 25.4, df = 5, $P < 0.01$; CMSE-FW = 3.87, df = 5, $P = 0.56$).

The QRS amplitudes in the treatment groups were comparable and not significantly different. The mean QRS amplitudes were CMSE-FW = 0.48 ± 0.04 mV, CMSE-CE = 0.55 ± 0.30 mV, and MS-222-CE = 0.59 ± 0.03 mV (Table 2). In addition, the QRS durations were comparable and not significantly different among the treatment groups. The mean QRS durations were CMSE-FW = 0.18 ± .02 s, CMSE-CE = 0.17 ± 0.01 s, and MS-222-CE = 0.13 ± 0.01 s (Table 2). However, the mean QT duration of CMSE-CE was significantly higher than the comparable values for CMSE-FW and MS-222-CE (Levine's test: $F_{2,27} = 5.51$, $P = 0.01$). The mean QT durations were CMSE-FW = 0.58 ± .01 s, CMSE-CE = 0.69 ± 0.01 s, and MS-222-CE = 0.57 ± 0.02 s (Figure 4; Table 2). Moreover, QT durations of CMSE-CE and MS-222-CE showed significant variation during the duration of the ECG procedure (Kruskal–Wallis test: CMSE-CE = 11.5, df = 5, $P = 0.04$; MS-222-CE = 20.7, df = 5, $P < 0.01$) but this duration was not significant for CMSE-FW (0.61, df = 5, $P = 0.99$).

### Respiration rates

The respiration rate of salmon was significantly reduced by CMSE-CE and CMSE-FW (hypopnea) and increased (tachypnea) by MS-222-CE (Levine's test: 13.37, df = 2, $P < 0.01$; *Supplemental Material*, Table S4; http://dx.doi.org/10.3996/032011-JFWM-025.S1). Before anesthesia, the respiration rate of salmon was 80 buccal pumps/min (pH 6.8, 12°C, 86% oxygen saturation, <10 mg/L salt). The respiration rate of CMSE-FW–anesthetized salmon was 15.2 ± 7 buccal pumps/min, of CMSE-CE–anesthetized salmon was 2.5 ± 1 buccal pumps/min, and of MS-222-CE–anesthetized salmon was 143.1 ± 63 buccal pumps/min (Table 2).

## Discussion

In this study, we found CMSE to be an effective immersion fish anesthetic. Salmon were rapidly anesthetized with CMSE

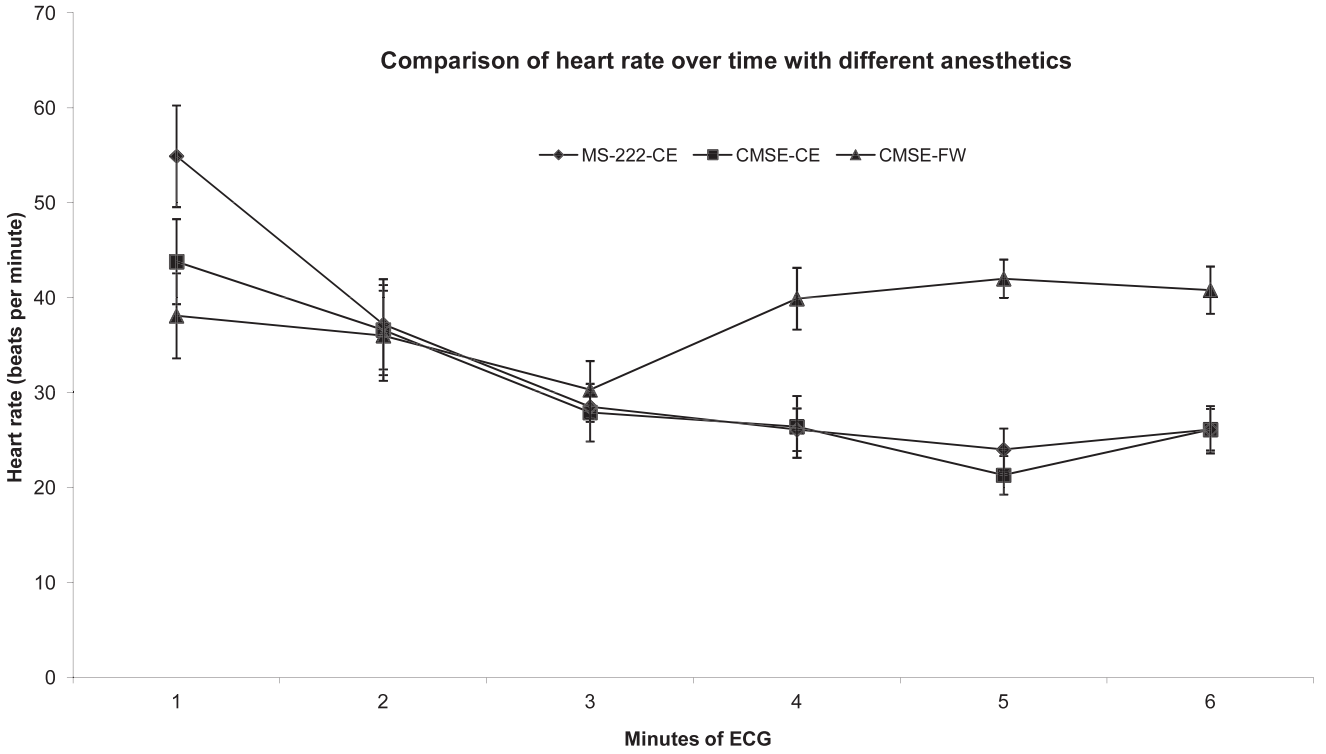


**Figure 3.** Heart rate (mean ± SE) of landlocked Atlantic salmon *Salmo salar sebago* exposed to an anesthetic for 2.5 min of induction to stage 5 anesthesia (i.e., loss of reactivity to tail pinch; opercular movements slow; heart rate slow; Brown 1993) and then attached to an electrocardiograph (ECG) for six additional minutes. Salmon anesthetized in tricaine methanesulfonate (MS-222) remained in the anesthetic solution during the ECG procedure (MS-222-CE). A second group of salmon anesthetized with the spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion (CMSE) fish anesthetic remained in the anesthetic solution during the ECG procedure (CMSE-CE), whereas the third group of CMSE–anesthetized salmon was transferred into fresh water for the ECG procedure (CMSE-FW).

**Table 2.** Average per minute values (mean ± SE) for heart rate, QRS amplitude, QRS duration, and QT duration recorded during the electrocardiograph (ECG). Salmon anesthetized in tricaine methanesulfonate (MS-222) remained in the anesthetic solution during the ECG (MS-222-CE). Landlocked Atlantic salmon *Salmo salar sebago* were induced to stage 5 anesthesia (i.e., loss of reactivity to tail pinch; opercular movements slow; heart rate slow; Brown 1993) with spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion (CMSE) fish anesthetic or MS-222 for 2.5 min before beginning the ECG. One group of salmon anesthetized with CMSE remained in the anesthetic solution during the ECG procedure (CMSE-CE), whereas the other group of CMSE-anesthetized salmon was transferred into fresh water for the ECG procedure (CMSE-FW).

| Parameter (units) | Group | *n* | Avg. for each min during ECG (mean ± SE) | | | | | | Avg. |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | |
| Heart rate (beats/min) | CMSE-FW | 10 | 38 ± 6 | 36 ± 5 | 30 ± 3 | 40 ± 5 | 42 ± 5 | 41 ± 7 | 38 ± 2 |
| | CMSE-CE | 10 | 44 ± 4 | 37 ± 5 | 28 ± 3 | 26 ± 3 | 21 ± 2 | 26 ± 2 | 30 ± 2 |
| | TMS-CE | 10 | 55 ± 5 | 37 ± 5 | 28 ± 2 | 26 ± 2 | 24 ± 2 | 26 ± 2 | 33 ± 2 |
| QRS amplitude (mV) | CMSE-FW | 10 | 0.64 ± 0.15 | 0.52 ± 0.13 | 0.47 ± 0.10 | 0.49 ± 0.12 | 0.45 ± 0.09 | 0.34 ± 0.03 | 0.48 ± 0.04 |
| | CMSE-CE | 10 | 0.69 ± 0.11 | 0.55 ± 0.10 | 0.54 ± 0.10 | 0.52 ± 0.09 | 0.51 ± 0.10 | 0.52 ± 0.10 | 0.55 ± 0.04 |
| | TMS-CE | 10 | 0.65 ± 0.09 | 0.59 ± 0.07 | 0.58 ± 0.08 | 0.56 ± 0.09 | 0.60 ± 0.10 | 0.55 ± 0.09 | 0.59 ± 0.03 |
| QRS duration (s) | CMSE-FW | 10 | 0.20 ± 0.06 | 0.18 ± 0.06 | 0.20 ± 0.06 | 0.20 ± 0.04 | 0.16 ± 0.03 | 0.12 ± 0.02 | 0.18 ± 0.02 |
| | CMSE-CE | 10 | 0.24 ± 0.04 | 0.16 ± 0.02 | 0.17 ± 0.02 | 0.16 ± 0.02 | 0.17 ± 0.02 | 0.14 ± 0.02 | 0.17 ± 0.01 |
| | TMS-CE | 10 | 0.11 ± 0.02 | 0.13 ± 0.03 | 0.13 ± 0.03 | 0.13 ± 0.04 | 0.15 ± 0.04 | 0.13 ± 0.04 | 0.13 ± 0.01 |
| QT duration (s) | CMSE-FW | 10 | 0.60 ± 0.03 | 0.59 ± 0.04 | 0.59 ± 0.04 | 0.56 ± 0.04 | 0.57 ± 0.04 | 0.60 ± 0.03 | 0.58 ± 0.01 |
| | CMSE-CE | 10 | 0.68 ± 0.04 | 0.63 ± 0.03 | 0.69 ± 0.03 | 0.70 ± 0.03 | 0.76 ± 0.02 | 0.72 ± 0.03 | 0.69 ± 0.01 |
| | TMS-CE | 10 | 0.37 ± 0.05 | 0.58 ± 0.04 | 0.65 ± 0.03 | 0.58 ± 0.04 | 0.61 ± 0.03 | 0.64 ± 0.03 | 0.57 ± 0.02 |

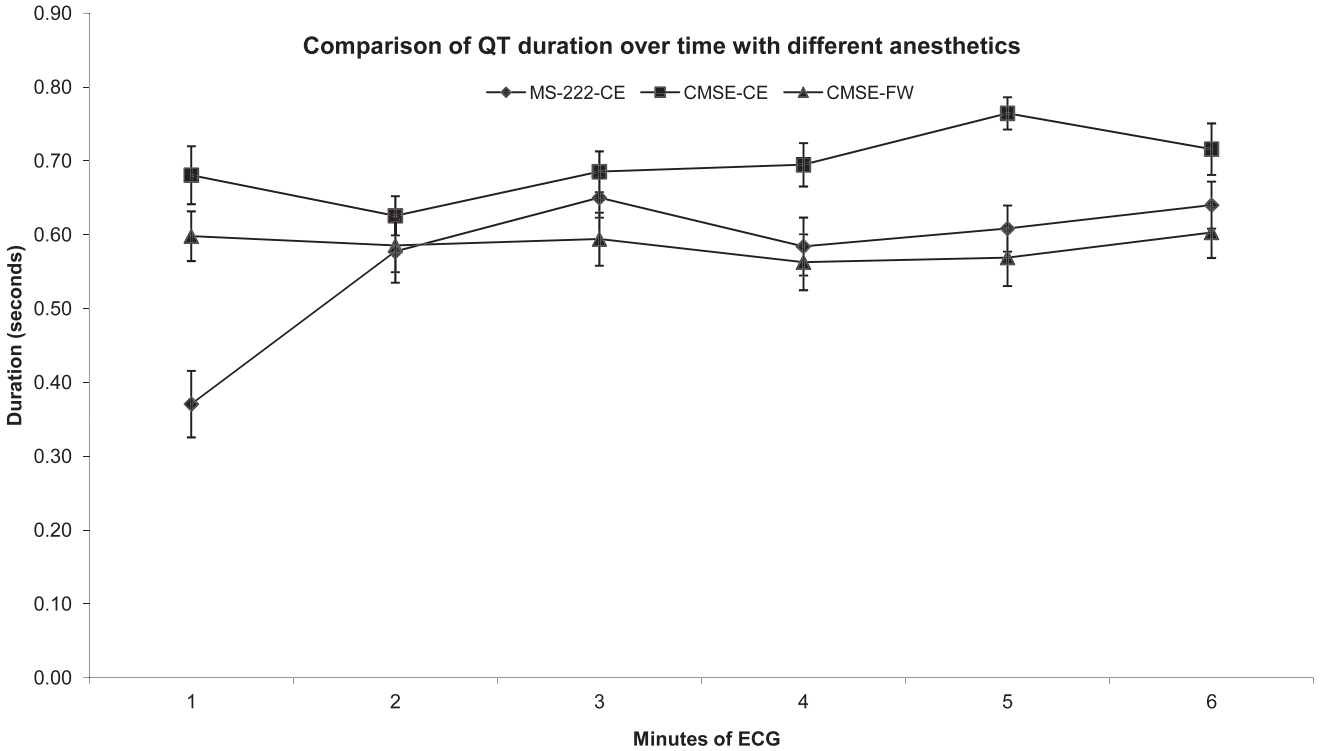


**Figure 4.** QT duration (mean ± SE) of landlocked Atlantic salmon *Salmo salar sebago* exposed to an anesthetic for 2.5 min of induction to stage 5 anesthesia (i.e., loss of reactivity to tail pinch; opercular movements slow; heart rate slow; Brown 1993) and then attached to an electrocardiograph (ECG) for six additional minutes. Salmon anesthetized in tricaine methanesulfonate (MS-222) remained in the anesthetic solution during the ECG procedure (MS-222-CE). One group of salmon anesthetized with the spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion (CMSE) fish anesthetic remained in the anesthetic solution during the ECG procedure (CMSE-CE), whereas the other group of CMSE–anesthetized salmon was transferred into fresh water for the ECG procedure (CMSE-FW).

to stage 4 anesthesia for routine husbandry procedures and were anesthetized to stage 5 anesthesia for an invasive procedure. All procedures were accomplished at concentrations of *l*-carvone and methyl salicylate that are similar to concentrations of spearmint oil and wintergreen oil found in human food, candy, chewing gum, tobacco products, toothpaste, mouthwash, cosmetic products, and approved pharmaceuticals. Because all of the ingredients in CMSE are already present in approved pharmaceuticals and the human food chain, CMSE may provide a solution to overcome the longstanding challenge of finding a 0-d preslaughter withdrawal anesthetic for food fish in the United States.

The efficacy of CMSE to anesthetize salmon is an important disclosure. Salmon are rapidly anesthetized to stage 4 anesthesia in approximately a minute of exposure to CMSE. Salmon remain anesthetized for several minutes after immersion when transferred into fresh water for human handling. This effect is advantageous for husbandry procedures that transfer batches of anesthetized fish to fresh water and thus prevents direct exposure of handlers to the anesthetic solution. The spearmint and wintergreen oil emulsion has potential as an immersion anesthetic for other salmonid species and many other cold-, cool-, and warm-water fishes that are important human food animals.

Salmon were anesthetized for an invasive ECG procedure with CMSE to assess the characteristics of stage 5 anesthesia achieved as well as the effects on the salmon heart's electrical activity. Salmon induced with CMSE and then transferred into fresh water for the ECG remained anesthetized for at least 6 min survived the procedure and had a stable heart rate, ECG pattern, and respiratory rate (Figure 3) and recovered without mortality. The effects of CMSE on salmon acid–base balance, hematocrit, blood gases and osmolality, and cortisol and adrenaline levels need to be determined. Salmon induced with CMSE and then kept in the CMSE solution during the ECG procedure progressed to deeper anesthetic depths as evidenced by progressive hypopnea and significant ECG abnormalities. The mortalities in the CMSE-CE and MS-222-CE study groups were not statistically significant and might have been due to the small size of salmon used, the acidic effects of unbuffered MS-222 used, or the minimal gill irrigation allowed. Additional studies controlling for these variables are needed.

Cotter and Rodnick (2006) published a similar ECG study comparing clove *Syzygium aromaticum* oil and MS-222. They were able to keep rainbow trout *Oncorhynchus mykis* immersed in either anesthetic solution for the duration of the ECG procedure without significant mortality. Cotter and Rodnick (2006) concluded that clove oil and MS-222 produced similar ECG patterns in anesthetized rainbow trout and that clove oil would be a

useful fish anesthetic. In contrast to clove oil and MS-222, CMSE has a much narrower therapeutic window for anesthesia beyond which CMSE can be fatal. Because there is no therapeutic labeled for euthanasia of fishes in the United States, CMSE potentially fills this gap in ichthyo-therapeutics as well.

Because the use of CMSE and its components as a fish anesthetic is not yet approved by the FDA, an FDA Investigational New Animal Drug (11-855) has been filed for CMSE as a food fish immersion anesthetic and euthanasia drug. Overall, CMSE is an effective anesthetic that is able to create stage 4 anesthesia in approximately a minute. It also creates and maintains a salmon in stage 5 anesthesia with only 2.5 min of exposure. Once induced, salmon can be transferred to fresh water for husbandry or invasive procedures; conveniently, salmon hold remarkably still and respire infrequently. An affordable anesthetic, made with readily available food ingredients and with a therapeutic range spanning between deep narcosis and fatally toxic, has many applications in aquaculture and fisheries for husbandry procedures; transport; and minor invasive procedures, such as radiotransmitter insertion, tagging, fin clipping tissue biopsy, and euthanasia. Overall, the combination of inducing variable levels of anesthesia, the lack of hypersensitivity or allergy to *l*-carvone in humans (Worm et al. 1998; Corazza et al. 2002; Surburg and Panten 2006), and its ergonomically useful features make CMSE a viable option as a 0-d preslaughter withdrawal and 0-d preangling, immediate release fish anesthetic.

## Supplemental Material

Please note: The *Journal of Fish and Wildlife Management* is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

**Table S1.** Detailed study design information, variables, statistics, assumptions, and power for stage 4 anesthesia experiment and stage 5 anesthesia with electrocardiograph experiment.

**Table S2.** Raw data detailing landlocked Atlantic salmon *Salmo salar sebago* sizes, anesthesia induction and recovery times, and survival for stage 4 anesthesia experiment.

**Table S3.** Comparison of recovery time from stage 5 anesthesia between the electrocardiograph cohort and control cohort.

**Table S4.** Data points from heart rate, QRS duration, QRS amplitude, QT duration, and respiration rate of landlocked Atlantic salmon *Salmo salar sebago* exposed to an anesthetic for 2.5 min of induction to stage 5 anesthesia (i.e., loss of reactivity to tail pinch; opercular movements slow; heart rate slow; Brown 1993) and then attached to an electrocardiograph (ECG) for six additional minutes. Salmon anesthetized in tricaine methanesulfonate (MS-222) remained in the anesthetic solution during the ECG procedure (MS-222-CE). A second group of salmon anesthetized with the spearmint (*l*-carvone) and wintergreen (methyl salicylate) oil emulsion fish anesthetic (CMSE) remained in the anesthetic solution during the ECG procedure (CMSE-CE), whereas the other group of CMSE anesthetized salmon was transferred into fresh water for the ECG procedure (CMSE-FW) as recommended by the CMSE drug label.

**Figure S1.** A sample electrocardiograph trace illustrating four heart beats with QRS amplitude, QRS duration, and QT duration labeled.

All found at DOI: http://dx.doi.org/10.3996/032011-JFWM-025.S1 (209 KB XLS).

## Acknowledgments

We thank the Ela Fish Rearing Facility, Embden, Maine, for providing the salmon and providing assistance during the ECG procedures conducted there; Austin Segel and the animal care staff at Colby College; and the Subject Editor and anonymous reviewers. The Colby College Institutional Animal Care and Use Committee approved the experiment in conjunction with a neurobiology course and undergraduate independent study research project.

Funding was provided by Colby College, Maine Department of Inland Fisheries and Wildlife, and the Maine Conservation Medicine Center.

The use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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