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EFFECTIVENESS OF THE ANESTHETIC AQUI-S® 20E IN MARINE FINFISH AND ELASMOBRANCHS

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ABSTRACT: Immersion anesthetics are used in hatchery settings by veterinarians, field biologists, and laboratory researchers to aid in handling finfish for medical procedures, research purposes, and moderating perceived stress responses. The only Food and Drug Administration– (FDA) approved anesthetic for food fish, tricaine methanesulfonate, requires a 21-d withdrawal period prior to harvest. Ten percent eugenol (AQUI-S® 20E) has been gaining momentum for FDA approval because of its 0-d withdrawal time if fish are not of harvestable size within 72 h of exposure. We performed two trials to determine appropriate anesthetic doses for two cultured marine finfish: *Atractoscion nobilis* (white seabass, WSB) and *Seriola lalandi* (California yellowtail, YT). Fish were held in a treated water bath for 10 min or until opercular beat rate slowed to a rate of <2 beats/min. Based on these results, we conducted a field trial with wild *Paralabrax maculatofasciatus* (spotted bay bass), *Paralabrax nebulifer* (barred sand bass), *Paralichthys californicus* (California halibut), *Triakis semifasciata* (leopard shark), and *Mustelus californicus* (grey smooth-hound) at a single dosing regime, with animals held 5–10 min in anesthetic baths. Anesthetic dosing of 35–55 mg L^{-1} provided relatively fast induction and good anesthetic maintenance in cultured and wild finfish. Anesthetic induction times were comparable among *S. lalandi* and *A. nobilis* at 35–mg L^{-1} to 75–mg L^{-1} doses, but recovery times were variable. Mortality rates of 20-90% were observed at higher doses (75 mg L^{-1} and 100 mg L^{-1}, *A. nobilis*; 55 mg L^{-1} and 75 mg L^{-1}, *S. lalandi*). The apparent increase in sensitivity of *S. lalandi* may have been associated with nutritional stress in the fish tested. There were no differences in time to anesthesia or recovery among wild finfish species tested at a single dose. Anesthetic induction, maintenance, and recovery were less predictable in the elasmobranch species tested and additional trials are needed to determine optimal dosing.

Key words: Anesthesia, animal welfare, AQUI-S 20E, elasmobranchs, finfish, marine.

INTRODUCTION

Anesthetics are used in clinical, research, hatchery, and field settings by biologists and veterinarians as a means of relieving perceived stress in aquatic animals for handling, and to offer pain relief during surgical procedures (Strange and Schreck 1978; Thomas and Robertson 1991; Iversen et al. 2003; Zahl et al. 2010). Perceived stress in fish has been reduced through the use of certain immersion anesthetics, which prevent plasma cortisol and glucose from rising above baseline levels (Iversen et al. 2003). Pain perception in aquatic fish is not fully understood, with some investigators concluding that fish experience pain as evidenced by increased gene expression in the forebrain during noxious stimuli (Reilly et al. 2008; Sneddon 2009), identification of nociceptors on the head of trout similar to higher vertebrates (Sneddon et al. 2003), the presence of opioid receptors in the nervous system, and diminished response to noxious stimuli when administered morphine (Sneddon 2003; Gonzalez-Nunez and Rodriguez 2009). Others conclude that fish lack conscious awareness of pain because they lack a neocortex, a structure unique to mammals from which consciousness arises, and because fish respond normally to stimuli when cerebral hemispheres are removed, with primary control coming from brain-stem and spinal cord structures (Rose 2002). However, increased animal welfare awareness has prompted the need for options for pain relief and anesthesia in fish, even if the mechanisms are not yet fully understood.

In wild and cultured food fish, there is a single Food and Drug Administration– (FDA) approved anesthetic, tricaine

methanesulfonate (MS-222, Western Chemical, Inc., Ferndale, WA), that can be used, but it requires a 21-d withdrawal time prior to harvest (Coyle et al. 2004). This limits its application in fieldwork with wild populations. Clove oil has been used experimentally for anesthesia in finfish (Kroon 2015) and contains components of methyleugenol, isoeugenol, and eugenol. Methyleugenol has been documented as carcinogenic in rodents, isoeugenol as inconclusive, and eugenol as having equivocal carcinogenicity results (FDA 2007). Fifty percent isoeugenol (AQUI-S®, AQUI-S New Zealand, Ltd., Lower Hutt, NZ) is not an FDA-approved anesthetic but has been associated with cortisol-reducing effects at concentrations ≥30 mg L^{-1} in salmon smolts after exposure to a stressor and during transport and transfer (Iversen et al. 2003). Its use was discontinued in the United States in 2008 because of human health concerns (Bowker and Pratt 2011). A modified version of the compound, 10% eugenol (AQUI-S 20E, AQUI-S New Zealand, Ltd., Lower Hutt, NZ) is currently being tested as an investigational new animal drug (INAD) in the United States. It is gaining momentum for FDA approval, and has a proposed 0-d withdrawal time if fish will not be of harvestable size within 72 h of exposure (AQUI-S 20E INAD 11-741). The potential stress- and pain-reducing capabilities for this new compound are still poorly understood in fish.

We manage a collaborative research and conservation program that releases juvenile *Atractoscion nobilis* (white seabass; WSB) in California coastal waters for stock enhancement purposes. We also culture *Seriola lalandi* (California yellowtail; YT). Here we report our efforts to determine the effectiveness of AQUI-S 20E as an anesthetic option in our cultured fish, and in wild fish and elasmobranchs encountered during field studies. Pilot studies were performed to determine appropriate dosing in our species, time to deep anesthesia as defined by Ross and Ross (1999), and time to recovery for short (10–15 min) procedures.

MATERIALS AND METHODS

We worked within the INAD program for AQUI-S 20E, and experimental designs were developed and approved in collaboration with the United States Fish and Wildlife Service (USFWS) Aquatic Animal Drug Approval Partnership Program (AADAP) administrators.

Trial 1

Seventy juvenile cultured WSB, age 116 d posthatch (dph), were randomly selected from the same crop and holding tank to use in the anesthetic trial. Ten fish each were individually immersed in a 3-gal treatment bath of AQUI-S 20E at 15–, 25–, 35–, 55–, 75–, and 100–mg L^{-1} doses, and in a 3-gal saline control bath. Treatment baths were changed once five fish had undergone treatment. Investigators performing observations were blinded to treatment and control doses. Time to stage II anesthesia (Ross and Ross 1999) and recovery were recorded for each fish. Stage II, plane II anesthesia was defined as the time when the fish lost its cough reflex, lacked muscle tone and equilibrium and did not respond to external stimuli, elicited by deep palpation of the lateral line in the peduncle region. Recovery was defined as the time when fish could swim in a purposeful manner, and perform two rotations around the tank avoiding obstacles. Opercular beat rate (OBR) was monitored continually and documented every 5 min. Fish were held in the treatment bath for a maximum of 10 min unless their OBR dropped below two per minute, at which time they were transferred immediately to an 18-gal recovery bath containing 33 parts per thousand (ppt) seawater. Air stones were present in both the treatment and recovery baths, and Hach HQ40d meter and probes were used to measure dissolved oxygen (DO), pH, and temperature (Hach Company, Loveland, CO) of source water at the start of the experiment and during each treatment. Hardness and salinity were recorded from the source water sample at the beginning of the experiment. At the end of each treatment period, total length (mm) and weight (g) were recorded for each fish, and fish were classified as not handleable, handleable, or anesthetized; fish that did not recover as expected were euthanized by an overdose of MS-222.

Trial 2

Fifty juvenile cultured YT, age 148 dph, were randomly selected from the same crop. Ten fish were individually immersed in a 3-gal treatment bath of AQUI-S 20E at 25–, 35–, 55–, and 75–mg L^{-1} doses, and a saline control bath.

Observers were blinded to treatment and control doses. All other methods were as listed for Trial 1.

Trial 3

Field trials: Twenty-three finfish or elasmobranches, commonly found in San Diego coastal waters, were collected from gill nets set for population surveys for anesthetic induction with AQUI-S 20E in preparation for surgical transmitter placement. Six *Paralabrax maculatofasciatus* (spotted sand bass), *Paralabrax nebulifer* (barred sand bass), and *Paralichthys californicus* (California halibut), two *Triakis semifasciata* (leopard shark), and three *Mustelus californicus* (grey smooth-hound) were caught and allowed to acclimate in net pens for 24 h prior to anesthetic induction.

Each teleost and elasmobranch was observed and behaviors recorded for 5 min prior to anesthetic induction. These observations served as our control. All fish and elasmobranchs were induced at a dose of 45 mg L^{-1} AQUI-S 20E in an 80-L static water bath supplied with air stones. Anesthesia and recovery were defined as described above for Trials 1 and 2. Subjects were placed on a surgical table for transmitter placement, supplied with a recirculating dose of 35 mg L^{-1} AQUI-S 20E at approximately 20 L min^{-1}. A total of 15 min was allotted for total anesthetic exposure time per the AADAP directives for this INAD. OBR or gill slit movement was continuously monitored and rate recorded prior to induction, at the end of anesthetic induction, end of the surgical procedure, and end of recovery. OBR or gill-slit rate and response to surgical incision (i.e., peduncle movement, muscle twitching) were used as indicators of a deep plane of anesthesia (minimum of stage II, plane II, Ross and Ross 1999) during transmitter placement. If response was noted, stimulus was withheld until subject returned to a deep plane of anesthesia. If a subject did not return to a deep plane of anesthesia, an additional 5 mg L^{-1} AQUI-S 20E was added to the recirculating anesthetic bath until a deep plane of anesthesia was reached. If OBR or gill slit rate dropped below two per minute, seawater was added to the recirculating anesthetic bath to reduce the concentration of AQUI-S 20E by 5 mg L^{-1}. At the end of each treatment period, total length (mm) and weight (g) of each animal were recorded and each subject put in an 80-L recovery bath with air stones. Time to anesthetic induction, surgery duration, and time to recovery were recorded.

Recovered juvenile spotted sand bass, barred sand bass, and California halibut were immediately released following the anesthetic procedure in compliance with the AQUI-S 20E FDA 0-day withdrawal time for fish not of harvestable size. Recovered leopard and grey smooth-hound sharks were held in net pens for 72 h postanesthesia in accordance with the experimental design developed in collaboration with AADAP administrators and the FDA. Water quality measurements of source water at the initiation of the experiment were as performed for Trials 1 and 2.

Analysis

For cultured fish trials (1 and 2), sample sizes were selected to allow determination of a 1-min difference among anesthesia and recovery times within and between species treated at various doses of AQUI-S 20E with 95% confidence. For the field study, sample size was selected to allow determination of a 2-min difference among anesthesia and recovery times among species with 95% confidence. The Shapiro-Wilk test was used to assess the data for normality. Data meeting normal distributions were analyzed via one-way analysis of variance (ANOVA), and post hoc Bonferonni pairwise comparisons were performed. Nonnormal mean rank data were analyzed via the nonparametric Kruskal-Wallis method, and Wilcoxon pairwise comparisons were performed between treatment groups and species with a $P<0.05$ used to infer statistical significance (StataCorp 12.0). Time to anesthesia and recovery data were further evaluated via Kaplan Meier time to event analysis to determine which anesthetic doses and species achieved a relatively rapid induction ($\leq$3 min), and a recovery within 30 min; subjects not meeting these time restraints were right-censored. Morphometric and mortality data were summarized by cultured species at each AQUI-S 20E dose.

RESULTS

Cultured species

The time to anesthesia and recovery distributions for cultured WSB are presented in Figure 1, with low doses requiring a longer time to anesthesia and shorter recovery time when compared with higher doses of AQUI-S 20E. WSB anesthetized at the two highest doses (75 and 100 mg L^{-1}) reached a deep plane of anesthesia (stage II, plane II) in under 2 min, and the lowest dose of AQUI-S 20E (15 mg L^{-1}) required 8 min to achieve anesthesia. Induction

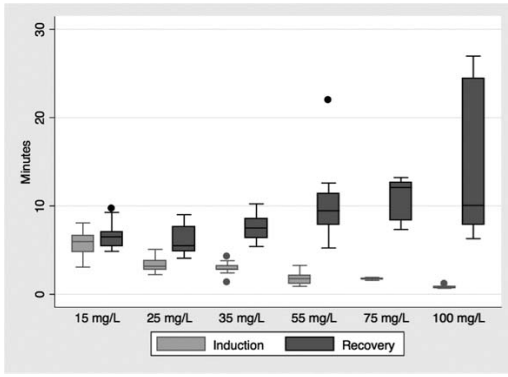

FIGURE 1. Box plots of *Atractoscion nobilis* (WSB) time to anesthesia (light grey boxes), and recovery (dark grey boxes) distributions by AQUI-S® 20E dose. Horizontal lines contained within the boxes represent median times; circles represent outliers (>1.5 times the lower or upper quartiles).

time ranks among doses were significant (χ^2=48.571, df=5, P=0.0001), but no differences among ranks were noted for time to recovery (χ^2=9.894, df=5, P=0.0783) in WSB. White seabass were more likely to achieve anesthesia in under 3 min when anesthetized at doses 55 mg L^{-1} and above (P<0.001), and were more likely to recover within 30 min when anesthetized at doses of less than 75 mg L^{-1} (P<0.01).

Anesthesia and recovery distributions for cultured YT are presented in Figure 2, with significant differences in time to anesthesia

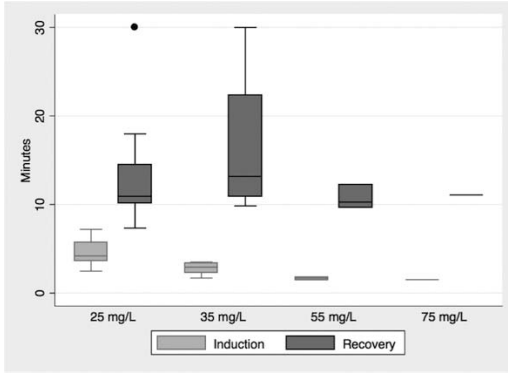

FIGURE 2. Box plots of *Seriola lalandi* (YT) time to anesthesia (light grey boxes), and recovery (dark grey boxes) distributions by AQUI-S® 20E dose. Horizontal lines contained within the boxes represent median times; circles represent outliers (>1.5 times the upper quartile).

(χ^2=28.418, df=3, P=0.0001) and recovery (χ^2=20.835, df=3, P=0.0001) among treatment ranks. California yellowtail were more likely to achieve anesthesia in under 3 min when anesthetized at 55 mg L^{-1} and higher (P<0.0001).

Time to anesthetic induction and recovery were compared between cultured WSB and YT species at the 25–, 35–, 55– and 75–mg L^{-1} doses. A significant difference in time to anesthetic induction rank was noted at the 25–mg L^{-1} dose (χ^2=4.166, df=1, P=0.0412), with YT taking a longer time to achieve anesthesia at this dose when compared with WSB. Differences in time to recovery ranks were noted at the 25– (χ^2=12.623, df=1, P=0.0004), 35– (χ^2=7.713, df=1, P=0.0055), and 55–mg L^{-1} (χ^2=5.733, df=1, P=0.0167) doses; recovery time in YT was longer when compared with WSB at the 25–mg L^{-1} and 35–mg L^{-1} doses, and shorter at the 55–mg L^{-1} dose. No differences were noted between WSB and YT with respect to dosing and ability to reach anesthesia within 3 min time (P>0.05), but YT were less likely than WSB to reach recovery within 30 min at the 25–mg L^{-1} and 35–mg L^{-1} doses (P<0.0001).

WSB and YT demonstrated a smooth induction and recovery at lower dosing regimes, with excitement phases (as evidenced by rapid swimming around the induction tank) noted in the 75–mg L^{-1} and 100–mg L^{-1} doses for WSB and 55–mg L^{-1} and 75–mg L^{-1} doses for YT. Higher dosing affected survival despite quick intervention at the first sign of distress (i.e., pulling cultured fish from the treatment bath prior to the 10-min end treatment when OBR decreased to <2 beats/min). California yellowtail demonstrated higher mortality at the 55–mg L^{-1} and 75–mg L^{-1} doses when compared with WSB, despite those fish having a higher average body weight and less anesthetic exposure time (Table 1). Normal swimming and respiratory behavior was noted throughout for all control fish.

TABLE 1. Mean weight (Wt), mean total length (TL), sample size, number of fish undergoing partial treatment (truncated because of anesthetic complications), and percent mortality for cultured *Atractoscion nobilis* (WSB) and *Seriola lalandi* (YT) treated with AQUI-S® 20E at various doses.

Species	Dose (mg L^{-1})	Mean wt[a] ± SE (g)	Mean TL[a] ± SE (mm)	*n* sample	*n* partial treatment	Mortality % (95% confidence interval)
WSB	15	36.9 (±1.75) A	161.0 (2.74) A	10	0	0
	25	37.4 (±2.08) A	161.3 (2.44) A	10	1	0
	35	39.6 (±3.37) A	164.7 (3.60) A	10	0	0
	55	35.2 (±2.57) A	158.6 (4.15) A	10	6	0
	75	34.6 (±1.67) A	156.6 (2.35) A	10	4	50 (0.167–0.832)
	100	31.0 (±1.82) A	153.4 (2.60) A	10	10	20 (−0.066 to 0.466)
YT	25	60.9 (±6.05) A	179.1 (4.58) A	10	6	0
	35	131.9 (±9.43) B	239.2 (8.20) B	10	1	10 (−0.101 to 0.301)
	55	106.7 (±11.98) B	218.2 (12.57) B	10	10	70 (0.393–1.007)
	75	137.1 (±7.40) B	231.4 (4.12) B	10	10	90 (0.699–1.101)

[a] Significant ANOVA differences within species are denoted by different letters.

Wild species

Wild finfish ranged from 290-mm to 534-mm total length, and 40–160 g. Elasmobranch size range was from 537-mm to 899-mm total length and weight range from 70 g to 300 g. Mean anesthetic exposure time was 6 min 54 s for finfish, and 19 min 47 s for sharks.

Time to anesthesia and recovery (Fig. 3) did not vary among wild finfish species ($\chi 2=1.556$, df=2, $P=0.4594$). Similarly, no differences were noted in time to anesthesia or recovery when comparing times among elasmobranch species, though sample size was small. Elasmobranchs were less likely to achieve anesthesia within 3 min than the finfish species ($P<0.04$) and finfish were more likely to recover within 30 min than elasmobranchs ($P<0.04$). Elasmobranchs had an anesthetic induction time twice as long and recovery time approximately three times as long as finfish species. Differences in behavior were noted, with finfish exhibiting relatively smooth induction and recovery and elasmobranch species exhibiting a prolonged induction, with more of an excitement phase, as evidenced by rapid swimming and occasional twitching behavior noted in grey smooth-hounds. The smooth-hounds were less likely to maintain a surgical plane of anesthesia when stimulated. All finfish and elasmobranchs recovered from the anesthetic procedure. One grey smooth-hound aborted pups 24 h following the anesthetic event.

Water quality

Water-quality values documented were within expected parameters for each trial (Table 2).

DISCUSSION

In our hatchery setting, MS-222 is used regularly to assist with handling and tag implantation as part of a WSB conservation stock enhancement program. Because of its extended withdrawal time, we sought to determine if an investigational new animal anesthetic, AQUI-S 20E, would be effective for use in a variety of cultured and wild finfish and elasmobranch species in both the hatchery and field setting.

We found that a dose range of 25–55 mg L^{-1} provided an effective plane of anesthesia (stage II, plane II, Ross and Ross 1999) for a 10-min procedure in our cultured WSB with no associated postanesthetic mortality. White seabass recovering from the 100–mg L^{-1} dose appeared to recover faster than at the 75–mg L^{-1} dose, but this likely is due to shortened treatment times because of observed respiratory depression at the higher dose. In cultured YT species, the safe and effective dose

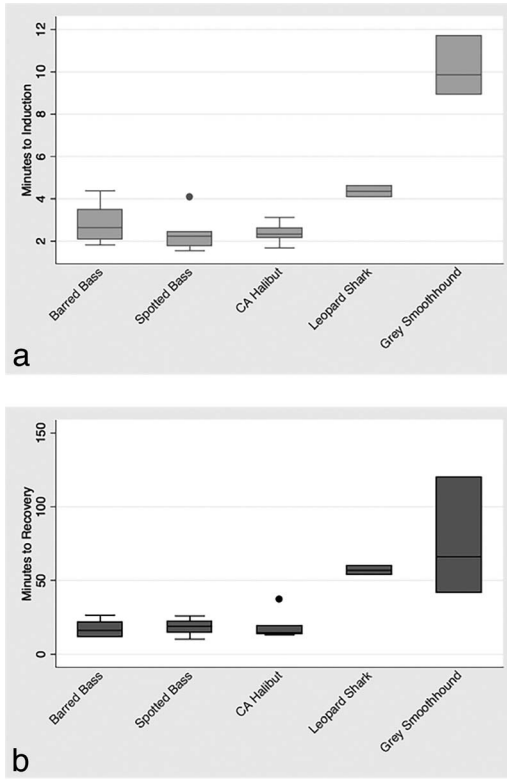

FIGURE 3. Box plots of time (min) to anesthesia (a) and recovery (b) distributions for wild *Paralabrax maculatofasciatus* (spotted sand bass), *Paralabrax nebulifer* (barred sand bass), *Paralichthys californicus* (California halibut), *Triakis semifasciata* (leopard shark), and *Mustelus californicus* (grey smooth-hound) induced at a 45-ppm dose of AQUI-S® 20E. Horizontal lines contained within the boxes represent median times; circles represent outliers (>1.5 times the upper quartile).

range was narrower, 25–35 mg L^{-1}, with significant mortality noted at 55 mg L^{-1} and above.

There was more size variability in the YT we anesthetized, perhaps as a result of a previous nutrition trial (1 mo prior). This variability introduces a possible confounding factor (body condition) with our YT results, particularly at higher doses. We attribute the shorter recovery time seen at the 55–mg L^{-1} dose in YT to truncated treatment (times <4 min of anesthetic exposure) because of observed apnea.

Based on our work with cultured fish, we chose a midrange dose of 45 mg L^{-1} for the field study, where we were seeking smooth induction, a consistent plane of anesthesia for a short surgical event (transmitter implantation), and uneventful recovery. All of the wild finfish studied had similar times to induction (<3 min) as seen in our cultured WSB and YT in the 35–mg L^{-1} to 55–mg L^{-1} dose range. Wild finfish had a smooth induction, as evidenced by minimal increase in swim speed and an ease of surgical positioning (ventral side up). Recovery was also smooth, with a slow, progressive return to peduncle movement and swimming around the recovery tank.

Elasmobranchs had a longer time to anesthesia and recovery than all of the finfish species studied. Opportunistic sample size achieved was too small to demonstrate significance, but the trend suggests that the drug acts differently in elasmobranchs. Elasmobranchs had more of an excitement phase, as evidenced by a rapid swim speed around the treatment tank, with an occasional twitch behavior when undergoing induction. This was particularly evident in all of the grey smooth-hounds studied. Similarly, juvenile *Entosphenus tridentatus* (Pacific lampreys) have demonstrated adverse leaping behaviors when exposed to AQUI-S 20E anesthesia (Christiansen et al. 2013), which may indicate that this drug has species-specific induction effects. Furthermore, all elasmobranchs appeared to

TABLE 2. Water-quality parameters measured at the start of each trial (salinity, alkalinity) and those measured throughout each trial (temperature, dissolved oxygen: DO, pH; means ± SD) are summarized.

Species/trial	Temp (C)	DO (mg L^{-1})	pH	Salinity (ppt)	Alkalinity (mg L^{-1})
WSB/Trial 1	22.0 ± 0.64	8.22 ± 0.126	7.65 ± 0.091	33.0	170.4
YT/Trial 2	20.7 ± 0.37	8.64 ± 0.446	7.94 ± 0.146	33.4	105.2
Wild/Trial 3	20.1 ± 1.36	8.34 ± 0.198	7.99 ± 0.101	33.7	120.7

be in a lighter plane of anesthesia (stage II, plane I, Ross and Ross 1999) when initially beginning surgical stimulation and required additional time without stimulation to achieve the desired plane of anesthesia. This resulted in a total anesthetic exposure time of slightly longer than 15 min in the grey smooth-hounds, which is the maximum exposure time for which AQUI-S 20E is currently labeled during investigational use.

This was the first time AQUI-S 20E has been used on wild elasmobranch species in the United States, and thus guidance on dosing was based on results in sharks (Frick et al. 2009, unpublished international colleague dosing reports) with a similar drug (AQUI-S, 50% isoeugenol, New Zealand Ltd.). Grusha (2005) reports successful use of clove oil (includes active ingredients 85–90% eugenol and variable isoeugenol and methyleugenol concentrations) in other elasmobranchs, cownose rays, at a dose of 50 mg L^{-1} successfully. It is possible that a higher dose of AQUI-S 20E, which contains 10% eugenol (compared to 50% isoeugenol tested by Frick et al. 2009) is required to result in a safe, rapid induction and steady anesthetic maintenance for elasmobranch species. However, a higher dose would amplify concern for prolonged recovery times as the elasmobranch species we exposed to AQUI-S 20E had longer recovery times than the finfish species. This may have been due to increased exposure time to AQUI-S 20E, but other variables should be investigated.

Although all wild finfish and elasmobranchs recovered from anesthesia, one grey smooth-hound whose pregnancy was not detected during handling aborted pups 24 h following the procedure. The other two grey smooth-hounds, which appeared to recover completely from anesthesia, were found dead 48 following the procedure. We were unable to determine cause of death, although sharks were thin, and this may have affected their ability to overcome stressful events (i.e., anesthesia, post-anesthetic holding period). Alternatively, the species may be intrinsically more susceptible to the drug. Skin thickness and scales have been reported to affect the uptake of immersion drugs through skin diffusion (Ferreira et al. 1984), and differences in integument between fish and elasmobranchs may have affected our findings in the species studied.

Although water quality was not the primary focus of this study, some routine values were recorded from the source tank at the start of each trial and during treatments. pH was of particular interest, given AQUI-S 20E is labeled to maintain a pH within 85% that of source water. Observations of pH measured during treatments did not vary by more than 0.04 units of each source water treatment bath. Compared to the acidic environment created by MS-222 (Welker et al. 2007), which requires buffering before fish treatment, AQUI-S 20E appears more neutral, thus lending itself to the smoother induction we observed. However, more in-depth monitoring of water-quality parameters at specified intervals would be necessary to provide statistical support for our anecdotal observations.

AQUI-S 20E appears to be a safe and effective drug for use in marine finfish anesthesia at low doses. Although our sample size of elasmobranchs was small and opportunistic in nature, several concerns emerged. One grey smooth-hound shark aborted pups. Two others recovered from anesthesia and looked well at 24 h but were found dead at 48 h postprocedure. Another concern with AQUI-S 20E in elasmobranchs is the longer exposure time for anesthetic induction, which resulted in longer recovery times. Better anesthetic monitoring, to include Doppler flow probe and ECG (Neiffer and Stamper 2009), might help explain the differences we observed between finfish and elasmobranchs. AQUI-S 20E appears to be a promising anesthetic option for field biologists given the current 0-d withdrawal time for fish not of harvestable size.

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