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Evaluation of Anesthesia Protocols for Handling Hogfish *Lachnolaimus maximus* using Tricaine Methanesulfonate and AQUI-S 20E®

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Abstract

Hogfish *Lachnolaimus maximus* are a high valued food fish with significant recreational and commercial fishing pressure and are a candidate species for marine aquaculture. There is a need to define safe and effective methods of anesthesia for handling of this species for aquaculture. Anesthesia efficacy was assessed with wild-collected adult (>20 cm, 0.2 to 1.2 kg) and juvenile F1 (<11 cm, 5 to 50 g) Hogfish, using tricaine methanesulfonate (Tricaine-S®) at 25, 50, 100, 125, and 150 mg L^{-1} and AQUI-S 20E® (10% eugenol) at 50, 75, 100, 200, 300, 400, and 500 mg L^{-1} to determine favorable doses for minor handling. Favorable doses resulted in induction of light anesthesia and recovery time each under 5 min, zero mortality, and limited excitation behavior. For adult Hogfish, Tricaine-S® was effective at inducing light anesthesia at 100-150 mg L^{-1} and was preferred over the effective range of AQUI-S 20E® doses (100-200 mg L^{-1}) based on fish behavioral observations while undergoing anesthesia. Additionally, induction of deep anesthesia was explored to inform potential doses for major and potentially lethal

procedures. These same ranges were effective at inducing deep anesthesia in adults. Juvenile fish were effectively anesthetized at the same doses of Tricaine-S® (100-150 mg L^{-1}) and were induced to light and deep anesthesia faster than adults at the same dose levels. AQUI-S 20E® was effective at inducing light anesthesia in juveniles at all levels tested, however no favorable dose for deep anesthesia was found. Overall, Hogfish were anesthetized with Tricaine-S® at similar doses used with other species and responded to AQUI-S 20E® similarly in terms of efficacy but unfavorably in terms of behavior.

Introduction

Hogfish *Lachnolaimus maximus* are a candidate for foodfish production. They are protogynous, monandric hermaphrodites with males achieving sizes close to 1 meter and 10 kg (Randall and Warmke, 1967). This species occurs in coastal waters of the Atlantic Ocean from North Carolina, south throughout the Gulf of Mexico, and as far south as Brazil (Lieske and Myers, 1994; Sampaio et al., 2016). They are common near rocky areas and reefs (Collins and McBride, 2011). Hogfish are a highly desired foodfish with an established market, targeted by both recreational and commercial anglers (Cooper et al., 2013). The predominant capture method for Hogfish is spearfishing. This harvest method is not readily scalable, which prevents Hogfish harvest from reaching the volume of other commercially targeted reef fish like snapper (Lutjanidae) and grouper (Epinephelidae), which are caught primarily by hook and line (NOAA, 2020). Compounding this is the reported overfishing of this species seen in regions of the Atlantic Ocean (Choat et al., 2010; Cooper et al., 2013). High demand exists for this species as a food product and supply levels may not be sustainable as overall reef fish density throughout the Western Atlantic continues to decline (Paddack et al., 2009; Pauly and Zeller, 2016; Zimmerman and Werner, 2019). Commercial scale production of other members of the wrasse family (Labridae) has been achieved (Grant et al. 2016) and initial larviculture of this species has proven feasible (Colin 1982). If captive Hogfish are to be maintained for commercial production, safe anesthesia protocols must be elucidated for necessary transport, tagging, and hormonal injection.

Chemical agents are used to anesthetize fish in a hatchery setting in preparation for close examination, hormonal injection, tagging, transportation, and measurement procedures (Ross and Ross 2008). Effective dose of immersion anesthetics may vary between species based on

physiology, respiration rate, and gill area to body weight ratio (Coyle et al., 2004). It is essential that proper species-specific dose levels are determined for an anesthetic agent to ensure health and safety of the fish and prevent losses (Trushenski et al., 2013). An ideal anesthetic outlined by Trushenski et al. (2012) is one that is safe for people and fish, effective at low doses in a predictable and rapid manner, has a high margin of error (i.e. wide range of doses before overdose occurs), allows for rapid recovery, and is inexpensive. This implies that optimum doses would have a timeframe of 5-10 min for induction and recovery to allow for the timely processing of fish (Silbernagel and Yochem, 2016). Safety of the chemical agent to both handlers and fish is paramount. The agent must not cause direct harm to handlers and should not lead to mortality or undue physiological stress on the fish. Certain anesthetic-specific considerations, which will be introduced in the following paragraphs, must be recognized. Further, a lack of sufficient anesthetic effect could cause indirect harm to both handlers and fish as many procedures occur out of water and involve sharp objects (e.g. needles, scalpels). The sudden, powerful movements of which fish are capable could lead to puncture, laceration, and/or impact injuries to fish and handler if the animal is not fully sedated. Therefore, proper selection of anesthetic agent and dosage are vital to safe, efficient hatchery practices.

The only FDA-approved chemical for sedation and anesthesia in foodfish is tricaine methanesulfonate (Tricaine-S®, Western Chemical, Inc., Ferndale, WA), which requires a 21-day withdrawal time before fish can be processed as food for human consumption (FDA 2019). By the definition put forth in Trushenski et al. (2012), there are several issues when using Tricaine-S® that make it less advantageous as the only legal option for anesthetizing fish. This chemical must be buffered in solution with sodium bicarbonate to balance its acidity and prevent harm to the fish's gill epithelia while in the anesthetic bath (Trushenski et al., 2013). Further, even when fully anesthetized, cortisol levels of fish may continue to rise, meaning fish may still be subjected to the physiological manifestations of stress even as they appear calm (Small, 2003; Coyle et al., 2004; Palić et al., 2006). Anesthesia addition alone may cause stress, even without handling (Smith et al., 1999). Captive broodstock often undergo repeated handling stress associated with normal culture procedures where anesthetization may be required or help reduce the impact of stress. Prolonged elevation of cortisol levels caused by such stressors has been shown to be immunosuppressive in fish (Palić et al., 2006). Long term immunosuppression can

adversely affect spawning quality and growth rates (Schreck et al., 2001; Tort et al., 2004), impacting production potential.

One alternative anesthetic agent that has been explored under the Investigational New Animal Drug (INAD) program is AQUI-S 20E® (10% Eugenol, AQUI-S New Zealand Ltd, Lower Hutt, New Zealand, INAD #11-741). Eugenol has been investigated as an anesthetic for a number of marine species including Cobia *Rachycentron canadum*, Yellowtail Jack *Seriola lalandi*, Seabass (*Atractoscion nobilis* and *Paralabrax* spp.), Halibut (*Hippoglossus hippoglossus* and *Paralicthys californicus*), Atlantic Cod *Gadus morhua*, Salmonids (*Salmo salar* and *Onchyrynchus mykiss*) and Elasmobranchs (*Triakis semifasciata* and *Mustelus californicus*) among others (Keene et al., 1998; Iverson et al., 2003; Zahl et al., 2010; Trushenski et al., 2012; Silbernagel and Yochem, 2016). Eugenol has been shown to limit stimulation of cortisol levels in blood plasma during anesthesia in some fishes (Iversen et al., 2003; Small, 2003; Palić et al., 2006) but not others (Zahl et al., 2010; Berlinsky et al., 2016). In Common Carp (*Cyprinus carpio*), eugenol was found to increase levels of circulating plasma aspartate transaminase (AST) and lactate dehydrogenase (LDH), which are general indicators of tissue damage (Yousefi et al., 2018). Induction and recovery times for sedation with eugenol vary with salinity for euryhaline fish (Barry et al., 2017), which could impact aquaculture operations working at reduced salinities. These properties suggest eugenol may be an advantageous anesthetic agent for use in marine finish aquaculture, however, species-specific concentrations and protocols should be defined.

The objectives of this research were to determine an effective dose range of Tricaine-S® and AQUI-S 20E® to induce light anesthesia for adult and juvenile Hogfish. Additionally, induction of deep anesthesia was explored to inform potential doses for major and potentially lethal procedures. Favorable doses were determined by low induction and recovery time (< 300 s), zero lethality, and limited excitation behavior. Ultimately, meeting these objectives would provide necessary information for practical hatchery management and future research with Hogfish.

Methods

Hogfish Collection and Anesthesia Methodology.- Adult wild Hogfish were collected by hook and line, net, and trap capture under the guidelines of a special activity license (SAL-19-2121B-

SR) issued by Florida Fish and Wildlife Conservation Commission. Fish were obtained from the Florida Keys and Tampa Bay area in March-July 2019 and were then transferred via hauling truck to a University of Florida Indian River Research and Education Center (UF-IRREC) outdoor greenhouse in Ft. Pierce, Florida. There fish were held in recirculating systems containing four 1600 L cylindrical black fiberglass tanks, a bead filter, protein skimmer, and supplemental aeration while undergoing a four-week quarantine. Frozen clams and shrimp were offered twice daily. After quarantine, fish were kept within these same systems for several months before experiments began. Bleach-sterilized Atlantic Ocean water was used for all saltwater applications at UF-IRREC and system water parameters were maintained at appropriate levels (salinity 30-35 g L^{-1}, 26-29°C, pH 7.9-8.3, DO >5 mg L^{-1}) and water quality (TAN 0 mg L^{-1}, Nitrite 0 mg L^{-1}, Nitrate <160 mg L^{-1}). Juvenile F1 Hogfish were produced at UF-IRREC from captive broodstock and were reared within a similar recirculating system to the adults containing a 1600 L blue polyurethane rectangular tank with a biofilter for 5 months prior to anesthesia treatment. Juvenile fish were approximately 6 months old at the time of the experiment and were being fed a pelleted diet multiple times daily. Fish used in this study were cared for under Institutional Animal Care and Use Committee (IACUC #201808719, University of Florida) protocols for proper handling and husbandry.

Anesthetic agent and dose efficacy were tested with two experiments on adult and F1 juvenile Hogfish. The anesthetic agents Tricaine-S® (Tricaine-S®, Western Chemical, Inc., Ferndale, WA) and AQUI-S 20E® (10% Eugenol, AQUI-S New Zealand Ltd, Lower Hutt, New Zealand, INAD #11-741) were used to determine effective doses for light anesthesia for minor handling of Hogfish. Minor handling was defined in this study as weighing, measuring, and potentially tagging fish. Deep anesthesia was also assessed to determine an effective dose for future procedures where light or surgical anesthesia would not suffice. Observed condition and classification of the level to which fish had been anesthetized was based on the common stage I-IV anesthesia with several planes of anesthesia at stage III (Ross and Ross 2008). A table that defines specific anesthesia stages can be found in Sneddon (2012). For this study, time points were recorded when fish experienced light anesthesia (stage III, plane I defined by loss of equilibrium) and deep anesthesia (stage III, plane III defined by cessation/rare gill movement), as well as recovery (stage 0, full return of motor function). Fish were determined to be in deep anesthesia at the first moment of total gill movement cessation.

Treatment baths were prepared in small (25 L) black polyethylene tubs and aerated with a single air diffuser. Water used for trials matched conditions of the systems in which fish were housed (30-35 g L^{-1} salinity; 23-27°C; pH 8.0-8.3) and the same treatment bath was used for all fish treated that trial day. For Tricaine-S®, the powder was weighed out to the desired concentration in mg L^{-1} and added to the bath with double the amount of sodium bicarbonate as a pH buffer. For AQUI-S 20E®, the liquid was measured using a graduated pipette to the desired concentration and added to the bath. All baths were aerated and mixed for 10 minutes prior to addition of fish. Fish selected for adult anesthesia treatments were considered at or near adult size (>20 cm) and ranged from 23 to 43 cm TL and 0.2 to 1.2 kg in weight. Juvenile fish (Collins and McBride 2011) ranged from 4 to 11 cm TL and 5 to 50 g. Fish were assigned to treatments to balance the range of weights of available fish among doses for as even a distribution as possible for each treatment. Fish were transferred from tanks to a temporary fiberglass holding tank (~100 L) for 5 minutes prior to treatments to standardize handling of fish prior to treatment. Fish had resumed normal breathing and swimming patterns before being added to the treatment bath.

Fish were individually placed into treatment baths. Any excitation behavior (defined here as jumping, thrashing, and behavior deviating from normal activity) was noted throughout the duration of each individual trial. The procedure was timed from when the fish was placed into the treatment bath, until they exhibited stage III plane I (loss of equilibrium) and stage III plane III (cessation of gill movement). These times were recorded. Once determined to be fully anesthetized, fish were handled and then placed into a recovery tank (~100 L, receiving pure oxygen injection) and timed to full recovery of motor function (stage 0). Dissolved oxygen levels in the recovery chamber were maintained at 100-150% saturation and monitored using a handheld YSI Multimeter (YSI inc., Yellow Spring, OH, USA). During temporal replication, the maximum number of juvenile fish and adult fish used in a single treatment bath was 15 and 8, respectively.

A total of 24 individual adult fish and 60 F1 juveniles were used for temporally replicated trials. Any fish receiving multiple anesthetic treatments was held more than 5 days between treatments under normal conditions of feeding and care. Individual fish were subjected to 2 to 4 rounds of treatment due to limited availability of appropriately sized fish. On a given trial day, one anesthetic agent was tested at three different concentrations with fish divided evenly among

treatment groups. Any fish that failed to reach either light or deep anesthesia, or recovery in under 600 s was given a time of 600 s. Due to the limited number of fish, those with no active gill movement after 30 s in the recovery bath were manually resuscitated by moving the fish through the water column to minimize mortalities.

Adult Hogfish Anesthetization.- Trials were conducted over four temporal replicates December 2019-January 2020. AQUI-S 20E® and Tricaine-S® were evaluated with adult Hogfish. Treatment baths of AQUI-S 20E® were prepared at concentrations of 50, 100 and 200 mg L^{-1} AQUI-S 20E®. Treatment baths of Tricaine-S® were prepared at concentrations of 25, 50, 100, 125, and 150 mg $L^{-1,}$ each buffered with sodium bicarbonate at an approximate 2:1 ratio so pH was equal to the fish's source water. Fish were kept in the treatment bath until they were determined to have reached deep anesthesia, or for a maximum of 600 s (10 min) for any fish that did not fully anesthetize. Induction times were recorded and fish were then removed, weighed, measured, and allowed to recover.

Juvenile Hogfish Anesthetization.- Juvenile F1 fish were acquired through volitional spawning of Hogfish broodstock maintained at UF-IRREC under photothermally manipulated conditions. Fish greater than 5 g were haphazardly selected for anesthesia treatment from a larger pool of juvenile fish. Trials were conducted over three temporal replicates October-November 2020. Treatment baths of AQUI-S 20E® were prepared at concentrations of 50, 100, 200, 300, 400, and 500 mg L^{-1} and baths of Tricaine-S® were prepared at concentrations of 50, 75, 100, 125, and 150 mg $L^{-1,}$ each buffered with sodium bicarbonate at an approximate ratio of 2:1 so pH was equal to the fish's source water. Data was collected and fish were handled identical to methods described previously.

Statistical Analysis.- All statistical analyses were performed in RStudio (R version 4.0.3). Normality was assessed visually with a Q-Q plot and quantitatively with a Shapiro-Wilk test. Homogeneity of variance was assessed visually by plotting residuals and by the global validation of linear models function (rpackage: gvlma) in RStudio. Any data not meeting assumptions was power transformed using a Box-Cox transformation. A one-way analysis of variance (ANOVA) was conducted on dose levels for each treatment chemical for either adult or juvenile fish. A post

hoc Tukey HSD test was performed when significant differences (α= 0.05) were detected among treatments. All measures herein are presented as mean ± SD to two significant figures.

Results

Adult Hogfish Anesthetization.- Adult fish treated with AQUI-S 20E® differed in weight (n=30, $F_{2,27}$=4.566, P=0.0196) with only the 50 mg L^{-1} treatment weighing less than the 150 and 200 mg L^{-1} treatments. Weight was significantly different because there was one large 1200 g individual and several smaller individuals. There were statistically significant differences in mean time to stage III plane I anesthesia among treatments (n=28, $F_{2,25}$=27.09, P<0.0001) with the 200 mg L^{-1} treatment having the shortest time to stage III plane I (Table 1). Statistically significant differences of mean time to stage III plane III were detected (n=30, $F_{2,27}$=122.8, P<0.0001), with all treatments being significantly different from the others. No fish treated at 50 mg L^{-1} AQUI-S 20E® was deeply anesthetized. Mean time to stage 0 varied significantly among doses (n=28, $F_{2,25}$=27.09, P<0.0001) with the 50 mg L^{-1} treatment again being significantly different from the 100 mg L^{-1} and 200 mg L^{-1} treatments. Excitation in the form of jumping was noted in 11 of the 30 individuals for AQUI-S 20E® (n=3 50 mg L^{-1}, n=3 100 mg L^{-1}, n=5 200 mg L^{-1}).

Mean weight of adult fish treated with Tricaine-S® did not vary significantly ($F_{2,27}$=0.888, P=0.423). No fish treated at either 25 or 50 mg L^{-1} Tricaine-S® were fully anesthetized (n=3 for both treatments) and those treatments were removed from further statistical analysis. Statistically significant differences in mean time to stage III plane I anesthesia were seen among treatments (n=30, $F_{2,27}$=7.2055, P=0.0031) with the 100 mg L^{-1} treatment having the longest time to stage III plane I. Significant differences in mean time to stage III plane III (n=30, $F_{2,27}$=31.55, P<0.0001) were also seen among treatments with all treatments differing significantly from each other. Mean time to stage 0 (n=30) varied significantly among treatments (P=0.0455) with the 100 mg L^{-1} treatment recovering faster than the 150 mg L^{-1} treatment. Excitation was noted in 8 of the 30 individuals treated with Tricaine-S® (n=3 100 mg L^{-1}, n=2 125 mg L^{-1}, n=3 150 mg L^{-1}). No mortality was seen in adult fish for either anesthetic at any dose tested.

Juvenile Hogfish Anesthetization.- Mean weight of juvenile fish treated with AQUI-S 20E® did not significantly differ between effective treatments (n=36, $F_{2,33}$=0.2336, P=0.793). No fish at 50

mg L^{-1} (n=3), 100 mg L^{-1} (n=5), or 200 mg L^{-1} (n=5) were deeply anesthetized and were removed from statistical analysis because of low sample size. Statistically significant differences in mean time to stage III plane I anesthesia were seen among treatments (n=36, $F_{2, 33}$=6.6671, *P*=0.0037) with the 300 mg L^{-1} treatment having a longer time to light anesthesia than both the 400 mg L^{-1} and 500 mg L^{-1} treatments (Table 2). There were no significant differences seen in either mean time to stage III plane III (n=36 $F_{2,33}$=2.60, *P*=0.0897) or stage 0 ($F_{2,33}$=2.9085, *P*=0.0687) among treatments. Some fish (n=4 300 mg L^{-1}, n=1 400 mg L^{-1}, n=1 500 mg L^{-1}) also failed to reach deep anesthesia at higher AQUI-S 20E® doses. Excitation was noted only in AQUI-S 20E® (n=1 300 mg L^{-1}, n=4 400 mg L^{-1}, n=6 500 mg L^{-1}) treatments. Nine fish in AQUI-S 20E® treatments (n=2 300 mg L^{-1}, n=4 400 mg L^{-1}, n=3 500 mg L^{-1}) had to be resuscitated with one mortality occurring in the 300 mg L^{-1} treatment. Of these, almost all took over 600 s to recover.

Mean weight of juvenile fish treated with Tricaine-S® did not significantly differ between effective treatments (n=45, $F_{2,42}$=0.0172, *P*=0.9829). Doses of 50 and 75 mg L^{-1} were ineffective at inducing deep anesthesia and were removed from statistical analysis. Statistically significant differences in mean time to stage III plane I were seen among treatments (n=45, $F_{2, 42}$=3.3163, *P*=0.046) with the 100 mg L^{-1} treatment having a longer time to light anesthesia than the 150 mg L^{-1} treatment. Mean time to stage III plane III was significantly different among treatments (n=45, $F_{2, 42}$=20.253, *P*<0.0001) with all treatments differing from each another. There were no significant differences in time to stage 0 seen among treatments (n=45, $F_{2, 42}$=0.1725, *P*=0.8422).

Discussion

No dose of AQUI-S 20E® tested on juvenile hogfish produced favorable results for safe and effective deep anesthetization, a state generally induced for potentially lethal procedures (Ross and Ross 2008; Sneddon 2012). With this chemical, juvenile hogfish seemed to be affected differently than adults, with many juveniles failing to deeply anesthetize even at a dose of 500 mg L^{-1}. Breathing remained very shallow but did not cease, sometimes for >9 min after reaching stage III plane I anesthesia. Mortality was seen in one individual, highlighting the risks of anesthetization past light anesthesia at the doses tested. However, light anesthesia was achieved in under 300 s for juvenile hogfish at all doses tested, although only treatments >300 mg L^{-1} were included in statistical analysis due to sample size. Two doses of AQUI-S 20E® (100 and 200 mg L^{-1}) fit the parameters of a favorable dose for adult Hogfish, characterized by light anesthesia,

deep anesthesia, and recovery times each under 300 seconds. The concentrations of AQUI-S 20E® in terms of eugenol content in experiment 1 were 5, 10, and 20 mg L^{-1}. In the literature, eugenol effectiveness has been explored as the product AQUI-S 20E®, as clove oil (70-90% eugenol), or as pure eugenol. Iverson et al. (2004) found that Atlantic Salmon would need a dose (~30 mg L^{-1} eugenol) that was at least 1.5x higher than what was necessary in experiment 1 for adult Hogfish to achieve either light or deep anesthesia. This same concentration of ~30 mg L^{-1} eugenol was found to be effective in Fathead Minnows *Pimephales promelas* over a slightly longer induction time (Palić et al., 2006). However, this was not found to be effective for juvenile Hogfish in this study. White Seabass and Yellowtail Jack were able to be induced to a similar state of deep anesthesia as was achieved in this study, but at much lower doses of AQUI-S 20E® (25-35 mg L^{-1}) with similar induction and recovery times (Silbernagel and Yochem, 2016). For both White Seabass and Yellowtail, full recovery took upwards of 30 min for some individuals, which is nearly 10x longer than the slowest recovery observed in adult Hogfish. This difference could be due to the methods of that study, in which anesthesia was prolonged at a lower concentration of eugenol once fish were induced prior to recovering (Silbernagel and Yochem, 2016). In this regard, it should be noted that recovery time after induction of deep anesthesia in hogfish may have been longer than recovery from light anesthesia alone. At 45 mg L^{-1} AQUI-S 20E® (4.5 mg L^{-1} eugenol) several species of seabass and California Halibut were able to be anesthetized in under 5 min (Silbernagel and Yochem, 2016), a dose which was shown to be ineffective for Hogfish even after 10 minutes. Alewives *Alosa pseudoharengus* were induced to surgical anesthesia at ~20 mg L^{-1} eugenol but authors recommended a ~40 mg L^{-1} dose as more effective (Berlinsky et al., 2016). Induction times for adult Hogfish at the 200 mg L^{-1} dose (20 mg L^{-1} eugenol) was similar to Alewife adults, and a 400 mg L^{-1} dose may be better suited for adult Hogfish and should be investigated as this produced acceptable outcomes for light anesthesia in juveniles. Ultimately, AQUI-S 20E® does not represent an ideal anesthetic for adult and juvenile Hogfish because of behavioral responses, safety concerns and current INAD restrictions, although its positive aspects include a fair range of effective doses and zero withdrawal time upon FDA approval.

No concentrations below 100 mg L^{-1} Tricaine-S® were effective at deeply anesthetizing juvenile or adult Hogfish. At every dose of Tricaine-S® juvenile fish were induced to both stages of anesthesia in a shorter time than adults and recovered in the same amount of time as the

lowest effective dose for adults (100 mg L^{-1}). Coyle et al. (2004) stated an effective concentration for rapid anesthesia of salmonids at 40 mg L^{-1}, which was not effective for Hogfish. This contrasts with the previously mentioned study by Iverson et al. (2004) in which salmonids would need close to 1.5x the maximum concentration of eugenol from experiment one for adult Hogfish to achieve deep anesthesia. It appears that Hogfish may be more sensitive to eugenol and less sensitive to Tricaine-S® than salmonids. Three doses of Tricaine-S® (100, 125, 150 mg L^{-1}) fit the parameters of a favorable dose for both juvenile and adult Hogfish as achieving light anesthesia, deep anesthesia, and recovery times each under 300 seconds. These effective doses coincide with similar effective concentrations for tilapia (*Oreochromis* spp.) (100-200 mg L^{-1}), Fathead Minnows (75 mg L^{-1}), and Channel Catfish (*Ictalurus punctatus*) (100-250 mg L^{-1}) (Smith et al., 1999; Coyle et al., 2004; Palić et al., 2006). The margin of safety at the doses examined was better compared to Fathead Minnows where 75 mg L^{-1} induced 100% of fish with 0% mortality but 100 mg L^{-1} led to 50% mortality (Palić et al., 2006). Overall Tricaine-S® represents a more ideal anesthetic for Hogfish on the basis of a range of effective doses, current legality, and limited excitation response, with a major drawback being the 21 day withdrawal time for human consumption.

Both anesthetic agents had doses resulting in favorable light anesthesia and recovery times (< 300 sec) for adult fish and juveniles. The lowest effective concentration of each anesthetic would be appropriate for inducing light anesthesia in Hogfish. Deep anesthesia was explored in this study because, as a novel and difficult to acquire species for captive aquaculture, broodstock Hogfish may need to undergo research procedures where this state is warranted. Exploring doses to induce deep anesthesia with a proper anesthetic agent establishes the bounds for safe treatment of Hogfish, where researchers can be aware of doses and timing where lethality can increase. For situations requiring deep anesthesia, researchers should opt for the lowest effective dose to increase the likelihood of fish survival when appropriate. The ineffective AQUI-S 20E® dose of 50 mg L^{-1} for adult experiments had a significantly lower mean weight of fish than the other two doses tested. A lower body weight would generally lead to sedative being more effective at a specific concentration (Bowker et al., 2015), meaning this dose would have likely remained ineffective even for larger adult Hogfish.

Both quantitatively and qualitatively, a clear difference between the two agents was the excitation observed in fish in the AQUI-S 20E® treatment. Adult fish experienced excitation

following exposure to both anesthetics, but fish in AQUI-S 20E® treatments were observed to retain this excited state for a longer time, although this was not explicitly quantified. For juveniles, this behavior only occurred in AQUI-S 20E® treatments. Many hogfish treated with AQUI-S 20E® were noted to be jumping out of the water during induction (n=11 for both experiments). Further, although adults were kept in the bath until cessation of gill movement, fitting the parameters of stage III plane III, many did not appear fully anesthetized during handling and would move with great force once or twice. For juveniles, AQUI-S 20E® seemed to depress breathing, but many times it did not fully cease even after 600 seconds.

The margin of safety for AQUI-S 20E® concentrations that induced deep anesthesia was not ideal for juveniles with nine fish needing to be manually resuscitated, one of which never recovered. No similar observations occurred for fish treated with Tricaine-S®. Interestingly, when placed into the recovery bath, fish that had not been deeply anesthetized in AQUI-S 20E® behaved as though they had been fully anesthetized with no control of equilibrium and longer recovery times than Tricaine-S® for both adults and juveniles. This was similarly noted by Coyle et al. (2004) as a difference between these two chemical agents with fish. These observations suggest that AQUI-S 20E® at these doses may be dangerous to both fish and handler, as fish struggled during and even after apparent anesthetization in adults and took longer to anesthetize and recover than Tricaine-S®. It is possible that the 200 mg L^{-1} AQUI-S 20E® dose was not high enough in adults and some of the more negative side effects of excitation would be lessened with a higher dose, although for juvenile fish excitation occurred more frequently in higher doses.

The pre-handling step all fish underwent may have also confounded results, as shown in Fathead Minnows where handling/crowding alone caused a similar blood cortisol elevation to that of MS-222 administration after 30 minutes (Palic et al., 2006). Blood cortisol levels were not examined in this study due to the scarcity of appropriately sized fish and dangers associated with repeated blood draws on broodstock and small juvenile fish in relation to immunosuppression and reproductive dysfunction (Thomas and Robertson, 1991; Pottinger et al., 1998; Palic et al., 2006). Due to temporal replication, some hogfish were exposed to the same anesthetic agent two times. Tilapia have been shown to have a sensitivity to weekly exposure to MS-222 (Tricaine-S®) after the third week (Smith et al., 1999). In this study hogfish were exposed to anesthetic agents as early as five days apart, but no more than four times over a 44 day period; the effect this had on results is unknown yet unavoidable due to a low number of available animals. Further

research may also find differences in recovery time if fish are induced to stage III plane I or II anesthesia alone.

This study defined effective doses for two anesthetic agents for both juvenile and adult sizes of Hogfish. For Hogfish, both AQUI-S 20E® and Tricaine-S® are efficacious at inducing light anesthesia, although Tricaine-S® appears to cause less excitation and should be preferred in most applications. Concentrations of Tricaine-S® in the range of 100 to 150 mg L^{-1} are effective and 100 mg L^{-1} should be used for most minor handling procedures of Hogfish. However, if AQUI-S 20E® were eventually FDA approved with its suggested zero withdrawal time, AQUI-S 20E® may become the preferred anesthetic for commercial producers selling food product at its effective dosage of 100 mg L^{-1}. Although not statistically analyzed due to lack of complete data, a dosage of 100 mg L^{-1} can tentatively be recommended for juvenile fish if AQUI-S 20E® is being used. Future research should evaluate other chemicals like Aquacalm® (Metomidate hydrochloride, Syndel Inc., CA, USA) and examine markers such as blood cortisol levels to better understand the physiological effects of stress that these anesthetics may cause on Hogfish. Further evaluations of AQUI-S 20E® at doses higher than 200 mg L^{-1} with adult Hogfish may also be warranted because of its proposed zero withdrawal period for foodfish. This would be advantageous if Hogfish were commercially cultured as a foodfish.

Table 1: Time (s) to stages of induction and recovery at various doses (mg/L) of two anesthetic agents for adult Hogfish *Lachnolaimus maximus*; *n* indicates sample size and an asterisk indicates treatments removed from statistical analysis from low sample size. Stages of anesthesia are based on Sneddon (2012). Different lowercase letters indicate post hoc significance among treatments for each anesthetic. The experiment was terminated at 600 s and instances of >600 indicate fish that never achieved that stage. Italicized recovery denotes recovery time from the deepest state of anesthesia achieved during the experiment.

Anesthetic	Dose (mg/L)	n	Stage III-I		Stage III-III		Stage 0		Weight		Excitation	Mortality
AQUI-S 20E	50	10	289.6 ± 103.1	b	>600	c	*103.8 ± 70.0*	a	352.7 ± 216.8	a	3	0
	100	10	102.5 ± 23.5	a	285.7 ± 94.2	b	181.2 ± 235.9	b	491.1 ± 185.8	b	3	0
	200	10	105.4 ± 35.8	a	208.3 ± 40.7	a	235.9 ± 50.8	b	683.4 ± 315.8	b	5	0
Tricaine-S	25*	3	600.0 ± 0.0		---		---		226.0 ± 19.2		0	0
	50*	3	150.0 ± 42.4		>600		*79.3 ± 36.7*		331.7 ± 71.2		0	0
	100	10	87.0 ± 47.8	b	261.1 ± 57.5	c	119.6 ± 34.0	a	507.7 ± 238.2		3	0
	125	10	58.1 ± 15.2	ab	168 ± 26.6	b	156.6 ± 66.0	ab	642.1 ± 271.7		2	0
	150	10	46.4 ± 9.0	a	128.6 ± 19.5	a	182.9 ± 71.4	b	642.0 ± 269.9		3	0

Table 2: Time (s) to stages of induction and recovery at various doses (mg/L) of two anesthetic agents for juvenile Hogfish *Lachnolaimus maximus*; *n* indicates sample size and an asterisk indicates treatments removed from statistical analysis from low sample size. Stages of anesthesia are based on Sneddon (2012). Different lowercase letters indicate post hoc significance among treatments for each anesthetic. The experiment was terminated at 600 s and instances of >600 indicate fish that never achieved that stage. Italicized recovery denotes recovery time from the deepest state of anesthesia achieved during the experiment.

Anesthetic	Dose (mg/L)	n	Stage III-I		Stage III-III		Stage 0		Weight	Excitation	Mortality
AQUI-S 20E	50*	3	103.7 ± 27.5		>600		*91.3 ± 81.6*		20.2 ± 8.5	0	0
	100*	5	55.2 ± 25.4		>600		*202.0 ± 52.9*		19.6 ± 10.6	0	0
	200*	5	38.0 ± 12.1		>600		*310.4 ± 145.1*		17.5 ± 13.1	0	0
	300	12	35.9 ± 8.7	b	450.8 ± 185.1		439.4 ± 182.0		27.8 ± 9.4	1	1
	400	12	28.7 ± 4.8	a	324.9 ± 127.3		431.9 ± 174.7		30.5 ± 18.1	4	0
	500	12	26.9 ± 4.9	a	298.4 ± 159.2		390.4 ± 180.2		32.1 ± 17.5	6	0
Tricaine-S	50*	3	115.7 ± 16.8		>600		*97.0 ± 19.1*		14.0 ± 12.4	0	0
	75*	3	112.1 ± 8.8		>600		*108.0 ± 6.7*		14.2 ± 11.8	0	0
	100	15	41.0 ± 7.5	b	195.3 ± 43.0	c	117.7 ± 29.8		22.8 ± 14.1	0	0
	125	15	38.9 ± 11.6	ab	148.4 ± 66.1	b	115.9 ± 32.2		23.6 ± 13.0	0	0
	150	15	33.4 ± 9.0	a	106.2 ± 22.2	a	119.2 ± 18.6		22.8 ± 12.9	0	0

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