Fish Physiol Biochem
https://doi.org/10.1007/s10695-020-00874-w

Anesthetic effects of AQUI-S 20E® (eugenol) on the afferent neural activity of the oyster toadfish (*Opsanus tau*)

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Received: 20 July 2020 / Accepted: 1 September 2020

Abstract Anesthesia is used to sedate aquatic animals during transportation or to immobilize them for surgery. However, most studies have focused on the behavioral effects of induction and recovery, without addressing the effect of anesthetic on neural activity. This study investigated the neural response of anterior lateral line afferent fibers in the oyster toadfish, *Opsanus tau*, during exposure to incremental increases of AQUI-S 20E (0.001–0.006%), to determine if eugenol (the active ingredient of AQUI-S 20E) influences neural activity of the fish lateral line system. Ventilation rate significantly decreased following AQUI-S 20E exposure with the surgical plane of anesthesia reached at 0.003%, characterized by shallow ventilation, equilibrium loss, and no response to tactile stimuli. Spontaneous and evoked firing rates of anterior lateral line fibers also significantly decreased following exposure, although the effect was transitory as neural activity recovered in the majority of fibers (70%) within 30 min of anesthetic withdrawal. While AQUI-S 20E proved effective in inducing the surgical plane of anesthesia without compromising survival, it is not recommended for acute neural preparations due to its depression of neural activity. However, the depression of lateral line sensitivity at low concentrations could play a role in reducing the stress response during fish transport.

Keywords Fish · Ventilation rate · Neural activity · Eugenol · Anesthesia

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10695-020-00874-w) contains supplementary material, which is available to authorized users.

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Introduction

Anesthesia is a biologically reversible state, induced by an external agent, and can be used to lightly sedate aquatic animals during transport and handling, or to immobilize them for surgical purposes by depressing their central and peripheral nervous systems (Javahery et al. 2012). Although the cellular mechanisms are unclear, it is hypothesized that some agents bind specifically to neuronal membrane proteins (such as Na^+ channels) and prevent the generation and conduction of nerve impulses (Levitan and Kaczmarek 2015; Armstrong et al. 2018), while other agents may act in GABA receptors affecting Cl^- channels (Garcia et al. 2010). In mammals, anesthesia results in the loss of sensory input, equilibrium, and proprioception (Summerfelt and Smith 1990). Although the effects of

Published online: 08 September 2020

anesthesia on nerve function in fishes are not well documented, fishes exhibit predictable behavioral changes that can be used to gauge the level of anesthesia (Zahl et al. 2012).

Several anesthetics are currently available for fishes including MS-222 (tricaine methane sulfonate), benzocaine, metomidate, and eugenol. In the USA, MS-222 remains the only legally registered anesthetic by the US Food and Drug Administration (FDA) for use in food fishes, and therefore it is widely used for fish transportation (Topic-Popovic et al. 2012) and commonly used for invasive surgical procedures (Priborsky and Velisek 2018). However, MS-222 has been associated with numerous side effects including hypoxia, elevated catecholamine and cortisol levels (King et al. 2005; Carter et al. 2011), and suppressive effects on peripheral and central neurons in acute preparations. The application of MS-222 at low concentrations (< 0.0050%) caused complete inhibition of the static discharge in response to the electrical stimulation of the ampullae of Lorenzini in dogfish (*Scyliorhinus canicula*) (Hensel et al. 1975). Neural activity in the lateral line nerve of African cichlids (*Tilapia leucosticta*) and common roach (*Rutilus rutilus*) were also reduced by > 70% following 3-min exposure to MS-222 (Späth and Schweickert 1977). Furthermore, a significant decrease in spontaneous and evoked activity of lateral line fibers was noted when anesthetic concentrations of MS-222 exceeded 0.0100% in the oyster toadfish, *Opsanus tau* (Palmer and Mensinger 2004). Inhibition of neural activity following anesthetic exposure presents a dilemma as the use of paralytics without anesthesia is considered unethical and therefore alternatives to MS-222, which could produce anesthesia with less effect on nerve function, would be valuable.

Eugenol (4-allyl-2-methanoxyphenol) is being considered as an alternative anesthetic for fishes because of its efficiency at a range of temperatures, availability, and low cost as well as safety for both fish and handlers (Javahery et al. 2012). Eugenol is a derivative of clove oil, a viscous liquid extracted from the leaves, buds, and stem of the clove tree, *Eugenia aromatica* tree. Eugenol is classified as GRAS (Generally Recognized As Safe) substance by the FDA for use in humans but has not been approved for use with fish in North America although eugenol is routinely used in other countries (i.e., Australia, New Zealand, Costa Rica, Republic of Korea and Chile) for sedating fish during transport (AQUI-S Ltd 2019). Eugenol has also proven to be an effective alternative for the sedation of larval, juvenile, and adult fishes [tambaqui, *Colossoma macropomum* (Roubach et al. 2005), guppy, *Poecilia vivpara* (Bolasina et al. 2017), and freshwater angelfish, *Pterophyllum scalara* (de Oliveira et al. 2019)] compared with other anesthetic agents available. However, based on behavioral observations during fish exposure to eugenol, such as equilibrium loss and reduced ventilation rate (Roubach et al. 2005; Bolasina et al. 2017; de Oliveira et al. 2019), it is hypothesized that neural activity will also be depressed. The challenge with testing the effect of anesthesia on neural activity in fishes is that fish need to be anesthetized for invasive procedures such as neurophysiological recordings and it is not possible to remove the anesthetic in acute preparation. While it is possible to implant electrode(s) for chronic neural recordings, it is challenging to maintain high-fidelity neural signals during implantation, recovery, and the subsequent re-exposure to anesthetic. An implantable micromanipulator has been developed to allow longer duration chronic recordings of neural activity in free-swimming fish to achieve this purpose (Rogers et al. 2017). The mechanosensory lateral line also provides a tractable model to determine the effect of eugenol on sensory systems. The lateral line sensory hair cells can be physiologically characterized and excited by external stimuli (Montgomery and Coombs 1998; Engelmann et al. 2002). Previous investigations in the oyster toadfish (*Opsanus tau*) have characterized the lateral line neurophysiology (Tricas and Highstein 1991; Palmer et al. 2005; Mensinger et al. 2019) and its response to MS-222 anesthesia (Palmer and Mensinger 2004). The overall aim of this study was to test whether eugenol influences the neural activity of fish sensory systems by recording spontaneous and evoked activity of afferent lateral line fibers in oyster toadfish exposed to increasing eugenol concentrations.

Materials and methods

Animal husbandry

Adult toadfish [25.1 ± 2.2 cm TL (mean ± 1 s.d.), 281.5 ± 72.8 g, $n = 16$ (10 males and 6 females)] were obtained from the Marine Biological Laboratory, Woods Hole, MA, and maintained in large, flow-through seawater tanks at ambient seawater temperature (19–21 °C) and salinity (29.5 ppt) (Supplementary Table 1). All

experimental procedures were approved by the Marine Biological Laboratory Institutional Animal Care and Use Committee.

AQUI-S 20E

This study was conducted under the Investigational New Animal Drug (INAD) Program (#11-741) regulations using AQUI-S 20E®and followed the guidelines for anesthetic administration required by the U.S Food and Drug Administration. AQUI-S 20E® is a commercialized clove oil product that is a certified anesthetic in Australia, New Zealand, Costa Rica, Republic of Korea, and Chile without a withdrawal period (https://aqui-s.com/products). The active ingredient in AQUI-S 20E is 10% eugenol [2-methoxy-4-(propenyl)phenol] (AQUI-S Ltd 2019). The protocol required a bath immersion with dosage range between 0.001 and 0.010%, that exposure to each concentration was limited to 15 min and fish were allowed to recover in anesthetic-free water.

Dose response curve

To assess the effect of AQUI-S 20E on toadfish behavior, control fish ($n = 9$) were individually transferred to rectangular (25 × 37 × 20 cm) aquaria, containing 7 L of aerated seawater (10 cm water depth). Following a 30-min acclimation period, 3.5 mL of AQUI-S 20E solution was incrementally added every 10 min to increase the dosage by 0.0005% up to 0.0040% (dose measurement calculations can be found in Supplementary Information). Following each addition, the water was stirred using a glass rod to mix the solution, and pH was measured (± 0.01) using a digital meter (Vantakool, MA, USA).

Anesthetic induction was assessed by measuring ventilation rate, equilibrium, and response to tactile stimulus using standard guidelines (Zahl et al. 2012) (Supplementary Table 2). The ventilation rate was determined as the number of opercular movements per minute. For each AQUI-S 20E concentration, equilibrium was assessed by inverting the fish with a net onto its dorsal surface and recording the time to return to its normal upright position. Tactile sensitivity was assessed by noting fish reaction to pinching the tail with forceps. Once the fish reached stage III-2 (surgical anesthesia), which is characterized by shallow ventilation, loss of equilibrium, and no response to tactile stimuli (Supplementary Table 2), it was transferred to another 7-L aquarium containing anesthetic-free seawater and monitored until it regained equilibrium (stage I) and resumed normal swimming (stage 0). All fish were subject to a second exposure 60 min after they resumed normal swimming, following the same experimental protocol. Fish were then euthanized with an overdose of 0.1% MS-222 and sex was determined via visual inspection of the gonads.

Microwire electrode and implantation

Microwire (three wire) electrodes were custom fabricated and integrated into an implantable micromanipulator (10 × 10 × 15 mm and 4.4 g) that was 3D printed using a Formlabs Form 2 3D printer (Somerville, MA, USA) (Rogers et al. 2017). The impedance of each electrode was determined with an impedance test unit (FHC Inc., Bowdoinham, ME, USA) prior to implantation and only electrodes with impedances between 0.5 and 1.0 MΩ were used.

Experimental fish ($n = 7$) were anesthetized by immersion in 0.005% MS-222 seawater solution and immobilized with an intramuscular injection of 0.01% pancuronium bromide (Sigma-Aldrich). Fish were secured within a stereotactic aquarium placed on a vibration isolation table, and a small dorsal craniotomy was performed to expose the anterior lateral line nerve. The implantable micromanipulator was secured to the dorsal surface of the skull using cyanoacrylate gel, and the microwires implanted into the anterior lateral line nerve anterior to its projection from the brain. Afferent neural activity was differentially amplified and monitored on a portable computer using Spike 2 for windows software (v. 8, Cambridge Electronics Design Ltd. Cambridge, UK). Two of the three microwire electrodes were chosen for recording based on signal fidelity. Once a candidate fiber was located, the fish was left undisturbed for 30 min to ensure fiber stability. The craniotomy then was sealed with cyanoacrylate gel and the muscle, fascia, and epidermis were sutured around the micromanipulator to create a watertight seal. Fish were then transferred to an experimental aquarium (25 × 37 × 20 cm) containing 7 L of aerated seawater, which completely immersed the fish and protruding micromanipulator (Fig. 1), and allowed to recover for at least 90 min, which is the time necessary for the MS-222 to lose its efficacy (Palmer and Mensinger 2004).

Experimental design

Following the recovery period, the microwire electrode tether was attached via a waterproof connector to a headstage (gain = ×10) connected to a differential amplifier (× 100; Dagan, Minneapolis, MN). A small brush was run over the surface of the fish to determine the approximate locations (± 0.5 cm) of the innervated lateral line neuromasts. A solid plastic sphere (15-mm dimeter) was attached to a mini shaker (Bruel and Kjaer, model 4810) by a 15-cm metal shaft and suspended vertically midway in the water column (5-cm depth) and positioned 5 cm from the innervated lateral line neuromast (Fig. 1). An externally triggered function generator (Tetronix FG 501a, Beaverton, OR, USA) was used to drive the mini shaker at 50 Hz [vertical displacement (peak-to-peak) = 0.05 mm].

The stimulus duty cycle consisted of 20 s on/ 20 s off, 50-Hz vibrational stimuli, and was repeated five times for each concentration. Spontaneous and evoked afferent firing rates were recorded, and waveform analysis used to discriminate individual units with Spike 2 software (v.8, Cambridge Electronic Design Ltd., Cambridge, UK). The spontaneous firing rate was defined as the number of spikes per second (spikes s^{-1}) between stimulus cycles while the evoked firing rate (spikes s^{-1}) was calculated during each stimulus presentation with five spontaneous and evoked firing rates averaged per concentration.

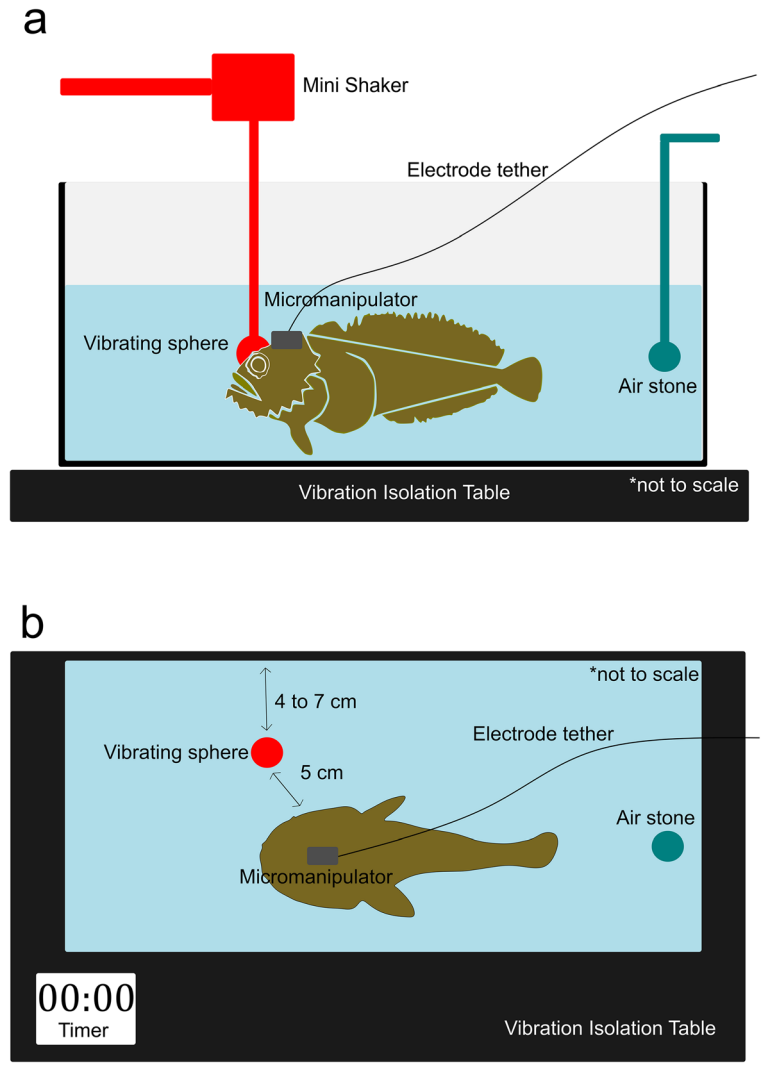

Fig. 1 Schematic representation of the experimental aquaria. **a** side view; **b** top down. The vibrating sphere was placed on the same side as the innervated lateral line neuromasts. Note: diagrams not to scale

At the start of the experiment, ventilation rate as well as spontaneous and evoked neural activity was recorded from the unanesthetized toadfish at three intervals, 10 min apart (T0, T + 10, T + 20). At the 30-min mark (T + 30), AQUI-S 20E was added to the aquarium and manually dispersed to produce an anesthetic concentration of 0.001%. After 5 min of exposure, the stimulus cycle was initiated, with ventilation rate, and spontaneous and evoked neural activity recorded. Additional AQUI-S 20E was then added at 10-min intervals to produce concentrations until the fish reached the surgical plane of anesthesia using the following timeline: 0.002% (T + 40), 0.003% (T + 50), 0.004% (T + 60), and if necessary 0.005% (T + 70) and 0.006% (T + 80), with spontaneous and evoked fiber activity recorded 5 min after each addition. Starting at T + 30, ventilation rate, response to tactile stimuli and pH was measured every 10 min. Once the fish reached stage III-3 anesthesia (Zahl et al. 2012) (Supplementary Table 2), the experimental tank was drained, rinsed, and refilled with anesthetic-free saltwater (7 L). Experimental fish were exposed to AQUI-S 20E anesthetic for a maximum of 90 min.

During recovery, the stimulus cycle was repeated every 10 min and ventilation rate, and spontaneous and evoked fiber activity were recorded, until the fish reached stage 0 (Zahl et al. 2012). Two experimental fish maintained high-fidelity signals in the same units ~ 12 h after the initial exposure and were subjected to a second AQUI-S 20E exposure. All experimental fish were euthanized with an overdose of 0.1% MS-222 and sexed via visual inspection of the gonads was determined.

Particle acceleration measurements

A calibrated waterproofed tri-axial accelerometer (Model: W356A12/NC; Sensitivity $X = 10.47$ mV/ms^{-2}; $Y = 10.35$ mV/ms^{-2}; $Z = 10.29$ mV/ms^{-2}; PCB Piezotronics, Depew, NY, USA) measured particle acceleration levels (dB re. 1 ms^{-2}) of the vibrational sphere during stimulus presentation. The accelerometer was made neutrally buoyant using polystyrene insulation sheathing and suspended in the middle of the water column 5 cm below the water surfaces and 5 cm lateral of the vibrating sphere. The tri-axial accelerometer was connected to a signal conditioner (Model: 482C; PCB Piezotronics, Depew, NY, USA) that amplified the signal (gain = ×100/axis) and was monitored with a PowerLab data acquisition system. All measurements ($n = 5$) were analyzed offline using LabChart software (Version 8; AD Instruments, Colorado Springs, CO, USA). Particle acceleration (dB re. 1 ms^{-2}) was calculated with a custom Matlab software (Version 2018a, Mathworks Inc., Natick, NA, USA) script, where the root mean square (rms) voltage (V_{rms}) values of each axis (x, y, and z axes) were corrected for the sensitivity of the accelerometer and then used to calculate the combined magnitude vector of particle acceleration in dB scale (Eq. 1).

$$dB\ re.1ms^{-2} = 20\log_{10}\left(\sqrt{(x^2 + y^2 + z^2)}\right) \quad (1)$$

Statistical analysis

All statistical analyses were performed using Sigmaplot (v14.0, Systat software). Data are presented as mean ± 1 s.d., and $p < 0.05$ was considered statistically significant for all analyses. All data was subjected to normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) testing and analysis that failed these tests were examined with non-parametric tests. To determine if there was a correlation between increasing the AQUI-S 20E concentration in the experimental aquaria and pH, a Person's correlation test was performed. A t test was used to compare the baseline ventilation rates (breaths per minute) at T0 between control and implanted toadfish.

Ventilation rates and lateral line firing rates (spontaneous and evoked) following exposure to AQUI-S 20E were normalized to the baseline rates determined prior to anesthetic exposure. Ventilation rates and firing rates were reported as percentages and arc-sin transformed prior to statistical analysis. To assess the effect of increasing AQUI-S 20E concentration on ventilation rate, a one-way ANOVA followed by a Holm-Sidak post hoc test was used separately for control and implanted toadfish. Additionally, the time (minutes) for ventilation rate to return to stage 0 from when the fish was placed in anesthetic-free water was compared between 1st exposure and 2nd exposure control toadfish, and between 1st exposure control and implanted individuals using a Mann-Whitney U test as the time data failed normality testing.

The correlation between lateral line fiber firing rate and concentration of AQUI-S 20E exposure was examined with a one-way ANOVA followed by a Holm-Sidak post hoc test for spontaneous and evoked firing rates. To determine the degree of phase locking of fiber

activity to the sinusoidal stimulus, phase histograms were generated for each unit. Using the first 500 spikes during the stimulus, the coefficient of synchronization (R) was calculated from the phase histograms to represent phase-locking strength. Strong phase locking is indicated as $R > 0.50$ and weak phase locking is represented by ≤0.50 (Goldberg and Brown 1969). It should be noted that the number of spikes at concentrations of AQUI-S 20E (≥ 0.005%) was insufficient to reach the 500-spike threshold; therefore, the R was calculated on a lower spike number. However, given that a small sample size (n) may misrepresent R, the Raleigh statistic (Z) was also calculated to determine whether phase locking was statistically significant ($Z > 6.91$; $p < 0.001$), where Z is a combined measure of the number of discharges (n) and the strength of phase locking (R) and is defined as $n \times R^2$ (Lu and Fay 1993). A Kruskal Wallis one-way ANOVA on ranks was conducted on coefficient of synchronization (R) values (as phase-locking data failed normality testing), to determine if there was a significance difference in phase locking due to concentration of AQUI-S 20E exposure.

Results

Normal swimming behavior was observed for all toadfish (control $n = 9$ and implanted $n = 7$) in anesthetic-free saltwater and all responded to tactile stimuli prior to anesthetic exposure. Ventilation rates were similar for each group, with no significant difference in ventilation rate of control [33.6 ± 3.8 breaths per minutes (bpm)] and implanted (33.9 ± 2.0 bpm) toadfish (Student's t test = − 0.175; $p = 0.863$) prior to AQUI-S 20E exposure. Additionally, water pH (7.91 ± 0.15) was not significantly changed by increasing AQUI-S 20E concentration (Pearson's correlation = 0.317, $p = 0.07$).

Behavioral effects of AQUI-S 20E

Control and implanted toadfish will be reported separately owing to slightly different experimental procedures: control toadfish were subjected to 0.0005% increments of AQUI-S 20E to determine the behavioral dose response curve while implanted fish were exposed in 0.0010% increments to minimize total exposure duration (Fig. 2).

Control toadfish exposed to 0.0005% AQUI-S 20E remained upright, continued to explore their aquaria, exhibited normal swimming behavior, and would right themselves in less than 5 s (stage I) (Table 1). At 0.001% AQUI-S 20E, fish were slower to return to an upright position (5 to 15 s) following inversion, and 5 out of 9 fish stopped swimming during the 10-min exposure. During 0.0015% AQUI-S 20E, activity increased (8 fish swam around the tank continuously), with fish struggling to regain equilibrium and often leveraged the sides of the aquaria to return to the upright position (stage II). Stage III-1 was attained at AQUI-S 20E ≥ 0.0020% and characterized by the partial loss of equilibrium with fish leaning to one side (Table 1). Stage III-2 was reached at 0.0030% AQUI-S 20E, with fish losing their reaction to tactile stimuli and remaining motionless (Table 1). All control fish reached stage III-3 at 0.0040% AQUI-S 20E, which was characterized by hyperextension of the operculum.

The implanted fish showed similar behavior at most stages; however, loss of equilibrium was not tested owing to the presence of the micromanipulator. Additionally, three of the implanted fish required higher concentrations of AQUI-S 20E (0.006%) to reach stage III-3 (Table 1). During the second exposure to increasing AQUI-S 20E concentrations [control ($n = 9$); implanted ($n = 2$)], behavioral observations also remained consistent (Fig. 2).

After transfer to anesthetic-free saltwater, all fish (control and implanted) resumed normal swimming behavior and regained their reaction to tactile stimuli (stage 0) within 60 min. There was no significant difference in the time (minutes) taken for control toadfish to recover to stage 0 following 1st and 2nd exposures (1st exposure control 9.4 ± 6.4 min; 2nd exposure control 11.1 ± 5.7 min) (Mann-Whitney $U = 31.500$, $p = 0.418$). However, there was a significant difference between 1st exposure control and 1st exposure experimental toadfish recovery time (1st exposure implanted 24.3 ± 16.8 min) (Mann-Whitney $U = 11.500$, $p = 0.031$).

There was a significant decrease in ventilation rate correlated with increasing concentration of AQUI-S 20E concentrations for both control (one-way ANOVA $F_{6,12} = 38.328$, $p = <0.001$) and implanted toadfish (one-way ANOVA $F_{6,12} = 38.328$, $p = < 0.001$), with significant decreases noted at ≥ 0.003% AQUI-S 20E concentrations [pairwise comparisons (Holm-Sidak test, < 0.05); Supplementary Tables 3 and 4]. For example, at 0.003% AQUI-S, control and experimental toadfish decreased gilling to 51.4 ± 11.0% (17.4 ± 5.1 bpm) and

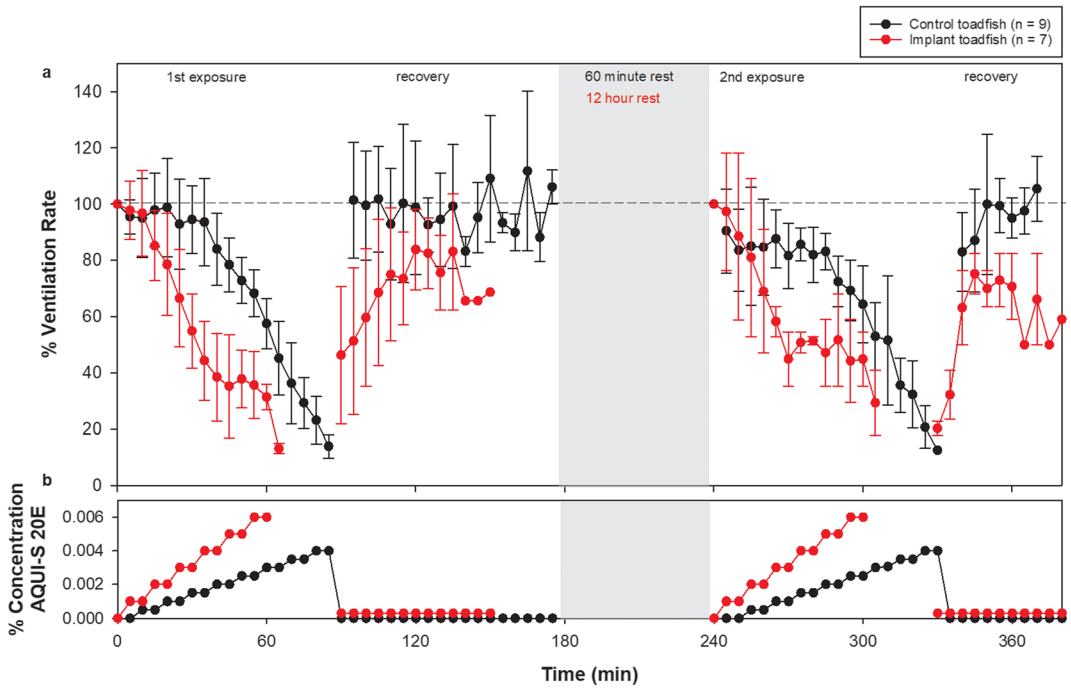

Fig. 2 Ventilation rate (as percentage of unanesthetized rate) of control toadfish (black; $n = 9$) and implanted toadfish (red; $n = 7$) during exposure to different concentrations of AQUI-S 20E and recovery. (**a**) Ventilation rate (%) shown as mean ± 1 s.d. Dashed line represents unanesthetized ventilation rate. (**b**) % Concentration of AQUI-S 20E in 7 L of aerated seawater with individual toadfish. Gray boxes represent the 60-min rest period for control toadfish and 12-h rest period for implant toadfish [Note $n = 2$ for 2nd exposure implant toadfish]

Table 1 Behavioral observations of control and implanted toadfish during AQUI-S 20E exposure

AQUI-S 20E concentration (%)	Stage**	Behavioral observation	Control ($n = 9$)		Implanted ($n = 7$)	
			Normalized ventilation rate		Normalized ventilation rate	
			1st exposure	2nd exposure (+ 60 min)	1st exposure	2nd exposure (+ 12 h)*
0.0000	0	No effect, normal swimming	100	100	100	100
0.0005	I	Delayed righting response (< 5 s)	96.5 ± 13.5	84.8 ± 19.0	NA	NA
0.0010	I	Delayed righting response (> 5 s), reduced swimming	95.8 ± 16.7	84.6 ± 10.9	97.0 ± 12.9	92.9 ± 25.3
0.0015	II	Struggling to regain equilibrium, increased swimming	94.0 ± 13.8	83.8 ± 7.9	NA	NA
0.0020	III 1	Partial LOE, decreased reaction to stimuli	81.2 ± 11.2	77.8 ± 7.7	81.8 ± 15.2	75.0 ± 25.0
0.0025	III 1	LOE, no swimming	70.6 ± 8.2	66.9 ± 12.2	NA	NA
0.0030	III 2	LOE, loss of reaction to tactile stimuli	51.4 ± 11.0	52.3 ± 17.6	60.7 ± 15.2	51.6.3 ± 7.5
0.0035	III 2	LOE, irregular breathing	32.8 ± 11.7	34.0 ± 10.8	NA	NA
0.0040	III 3	LOE, irregular breathing, hyperextension of operculum	18.5 ± 6.3	16.6 ± 3.7	41.4 ± 14.9	51.1 ± 2.6
0.0050	III 3		NA	NA	36.5 ± 14.3	49.5 ± 14.2
0.0060	III 3		NA	NA	33.5 ± 8.3	44.6 ± 12.2

Data represent mean ± 1 s.d. Ventilation rate is normalized as a percent of pre-anesthetized rates. *LOE* loss of equilibrium, *NA* not applicable

*2nd exposure of implanted fish conducted on two individuals

**Citation Zahl et al. 2012

60.7 ± 15.2% (17.4 ± 5.6 bpm) of the normalized baseline respectively (Table 1).

Effects of AQUI-S 20E on firing frequency

Ten anterior lateral line afferent fibers from seven fish were successfully maintained throughout the experimental parameters. Fibers were identified using the following nomenclature: TF 1 A = implanted toadfish 1 fiber A. An example of neural activity before and during stimulus presentation can be seen in Supplementary Information Fig. 1.

The mean pre-anesthetic spontaneous firing rate was 9.7 ± 7.5 spikes s^{-1} and evoked firing rate was 18.2 ± 10.6 spikes s^{-1} ($n = 10$ fibers). At stage III-3 anesthesia, firing rates decreased to 5.1 ± 8.5 spikes s^{-1} (spontaneous) and 6.2 ± 7.6 spikes s^{-1} (evoked) (Table 2). There was a significant decrease in both spontaneous (one-way ANOVA $F_{6,56} = 12.571$, $P < 0.001$) and evoked (one-way ANOVA $F_{6,56} = 7.468$, $P < 0.001$) firing rates that was correlated with increasing concentration of AQUI-S 20E for all units (Fig. 3a). At ≥ 0.001% AQUI-S 20E concentrations, evoked and spontaneous firing rates were significantly lower than baseline (pre-anesthetic) levels [Holm-Sidak post hoc test ($p < 0.05$)]. Additionally, at 0.006% AQUI-S 20E concentration, the spontaneous firing rates were significantly lower than at 0.001% and the evoked firing rates were significantly lower than 0.001 and 0.002% (Fig. 3, Supplementary Tables 5 and 6). Following transfer to anesthetic-free saltwater, spontaneous firing activity (11.2 ± 10.0 spikes s^{-1}) and evoked activity (21.8 ± 13.1 spikes s^{-1}) returned to pre-anesthetized firing rates in 19.1 ± 26.8 min and 22.2 ± 24.8 min respectively.

All fibers showed consistent decrease in firing rate in response to increased AQUI-S 20E; however, there was individual variation in response kinetics as shown by four fibers in Fig. 4. For example, TF 1 A firing rates decreased by a greater amount relative to the baseline (spontaneous 78.4%; evoked 77.1%) compared with TF 3 A (spontaneous 20.6% and evoked 26.2%) (Fig. 4a, g). Some toadfish also required a larger dosage than others to reach stage III-3 anesthesia, as toadfish 1 and 3 were exposed to AQUI-S 20E ≤ 0.004% and toadfish 4 and 6 were exposed to ≤ 0.006% (Fig. 4). TF 4 A showed a slight increase in evoked firing at 0.000 to 0.001%, followed by a continual decrease as concentration of AQUI-S 20E increased (Fig. 4b). Lastly, TF 6 A showed a decrease in both spontaneous and evoked firing rates (74.9% and 91.5% of pre-anesthetic rates respectively) as the concentration increased from 0.000 to 0.006% (Fig. 4j).

Particle acceleration levels increased at the fish position during the vibrating sphere stimulus (median = − 34.54 dB re. 1 ms^{-2}) compared with background levels (median = − 14.7 dB re. 1 ms^{-2}). Fibers showed varying phase-locking responses to the sinusoidal stimulus (Supplementary Information Fig. 2). Four fibers (TF 1 A, TF 4 A, TF 6 B, and TF 7 A) were strongly phase locked ($R > 0.50$ and $Z > 6.91$) during the pre-anesthetic baseline recording and all showed declines in phase locking as AQUI-S 20E concentrations increased (Fig. 5). The other fibers were weakly phase locked at the start and showed variable responses from remaining weakly phase locked to no longer exhibiting phase locking. Overall, there was no significant correlation between increasing concentration of AQUI-S 20E and phase locking (Kruskal–Wallis $H_6 = 2.081$, $P = 0.912$).

Table 2 Spike rates for the implanted toadfish

AQUI-S 20E concentration (%)	Spontaneous spike rate (spikes s^{-1})	Evoked spike rate (spikes s^{-1})
0.0000 (pre-anesthetic)	9.7 ± 7.5	18.2 ± 10.6
0.0010	6.9 ± 7.1	12.9 ± 10.4
0.0020	5.8 ± 6.3	10.9 ± 11.5
0.0030	5.1 ± 6.1	11.1 ± 12.4
0.0040	5.2 ± 7.4	9.7 ± 9.8
0.0050	6.0 ± 8.6	7.0 ± 7.3
0.0060	5.1 ± 8.5	6.2 ± 7.6
0.0000 (recovery)	12.3 ± 10.4	19.6 ± 10.6

Data represent mean ± 1 s.d.

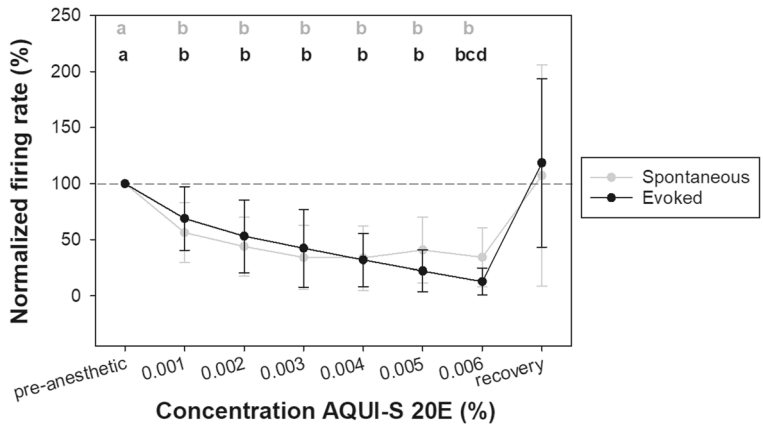

Fig. 3 Normalized firing rate of lateral line fibers ($n = 10$) plotted versus increasing AQUI-S 20E concentrations. Firing rate is normalized as a percentage of the pre-anesthetized firing activity. Data shown as mean ± 1 s.d. for spontaneous (gray) and evoked (black) firing rates. Different letters indicate statistical different groups (one-way ANOVA, $p < 0.05$)

Repeated AQUI-S 20E anesthesia effects and recovery

Two implanted fish were exposed to a second treatment of AQUI-S 20E following 12 h of recovery (Fig. 6). TF 2 required a higher AQUI-S 20E concentration during the second exposure to reach stage III-3 anesthesia (0.006% compared with 0.004%). However, both the time course of depression for ventilation rate and firing rate was similar during both exposure trials.

Discussion

Increased concentrations of AQUI-S 20E were correlated with decreased ventilation, sensitivity to tactile stimulation, and loss of equilibrium. There was a concurrent decrease in spontaneous and evoked firing rates of the anterior lateral line fibers, indicating that the anesthetic depresses neural activity. All fish successfully recovered from the exposure with ventilation rate and behavior returning to pre-anesthetic levels within 60 min and neural activity recovering in the majority of fibers (70%) within 30 min of anesthetic withdrawal.

MS-222 is the most commonly used anesthetic for fish surgical procedures; however, it has been associated with changes in hormone levels and suppressive effects on peripheral and central neurons in acute preparations (King et al. 2005). Alternatives to MS-222 that achieve similar levels of anesthesia, with less effect on neural activity, would have wide appeal for neurophysiologists. In this study, AQUI-S 20E (eugenol) yielded similar behavioral changes as those observed with MS-222 (Cho and Heath 2000). However, increasing concentrations of AQUI-S 20E significantly depressed lateral line fiber sensitivity. Even at relatively low concentrations (0.002%), while the fish remained responsive, firing rates decreased by up to 50%. In comparison MS-222 concentrations used to maintain surgical anesthesia (0.005%), had no significant change in the evoked firing rate, although spontaneous rates were depressed (Palmer and Mensinger 2004). Firing rate is not the only way to evaluate neural input, as phase locking in response to a low-frequency sinusoidal stimuli (< 1000 Hz) is considered an accurate way of evaluating the ability of the nervous system to encode stimuli (Köppl 1997; Schaus and Moehlis 2006). Strongly phase-locked fibers all decreased phase locking at the highest concentrations of AQUI-S 20E indicating the anesthetic not only depresses neural activity but may also interfere with signal encoding.

Invasive procedures present a challenge for sensory physiologists because ethical consideration requires anesthesia for restrained and/or immobilized preparation of animals, which may negatively impact the activity and sensitivity of the target system (Palmer and Mensinger 2004). Currently, the only option to examine the effects of anesthesia on neural sensitivity is to chronically implant electrodes under anesthesia, allow the fish to recover, and then challenge them again with anesthesia. In the current study, exposure to AQUI-S 20E was the second anesthetic agent experienced by the implanted toadfish because surgery was conducted under MS-222 with additional immobilization via the paralytic pancuronium bromide. Sufficient time (90 min) was allowed for the effects of MS-222 to dissipate (Palmer and Mensinger 2004) prior to initial AQUI-S 20E exposure. Both control (no previous anesthetic exposures) and implanted fish also showed similar behavioral responses, indicating that both the MS-

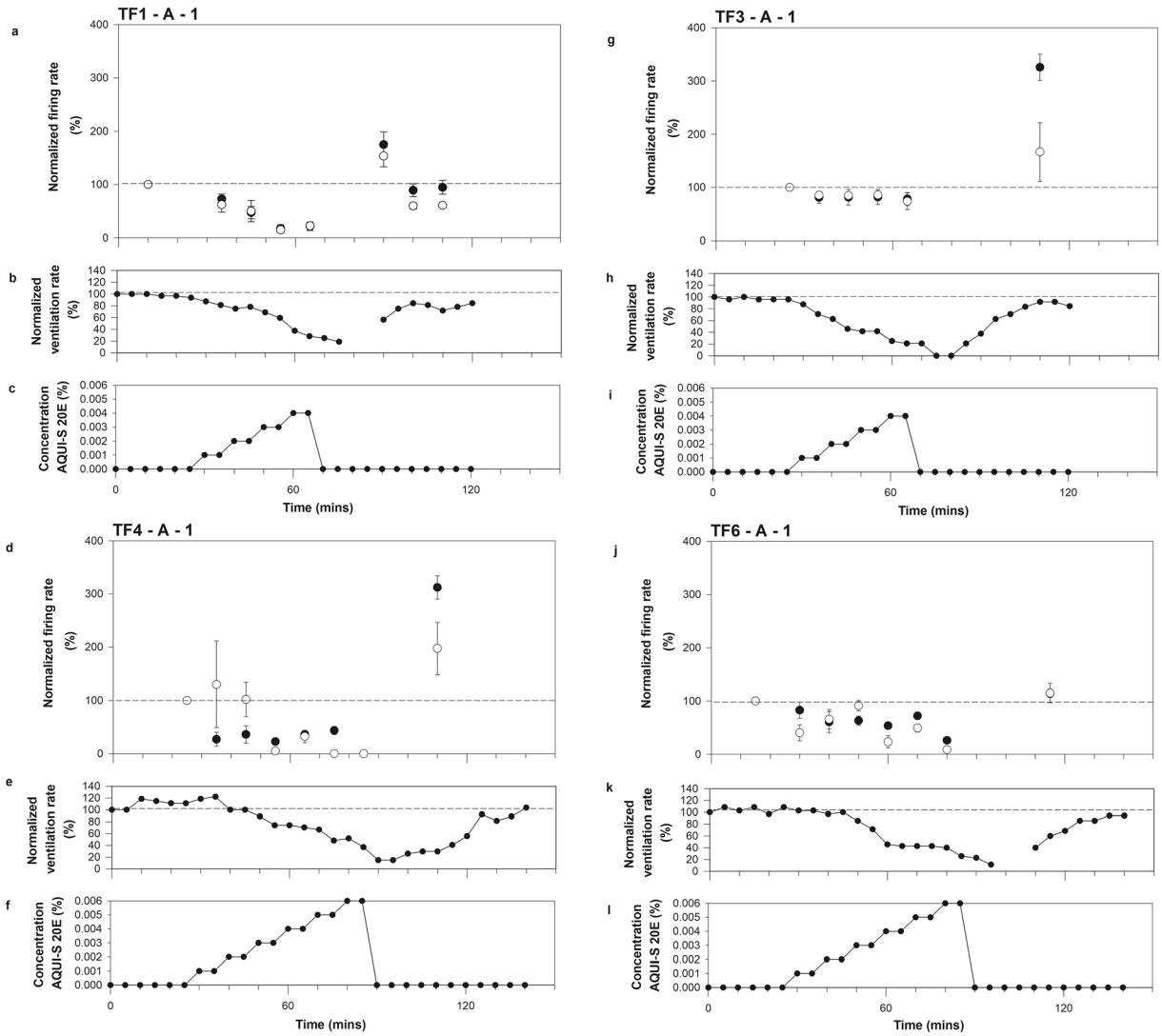

Fig. 4 Induction and recovery of lateral line units during application and withdrawal of AQUI-S 20E anesthesia for four toadfish. **a**, **d**, **g**, **j** Spontaneous (filled circles) and evoked (open circles) neural activity in response to anesthetic concentration is plotted versus time (min). Firing rate is normalized as a percent of pre-anesthetized firing activity. Data is show as mean ± 1 s.d. The dashed line represents the pre-anesthetized firing rate. **b**, **e**, **h**, **k** Ventilation rate of toadfish normalized as a percent of pre-anesthetized ventilation plotted versus time (min). **c**, **f**, **i**, **l** AQUI-S 20E concentration of the saltwater bath immersion for the toadfish plotted versus time (min)

222 exposure and subsequent electrode implant did not affect the reaction to AQUI-S 20E.

Occasionally there is also a need to re-anesthetize fish and reports indicate that inconsistent results can be encountered with repeated exposure. For example, African cichlid (*Tilpaia spilurus*) and roach (*Rutilus rutilus*) experienced a decrease in neural response to repeated exposure to MS-222 (Späth and Schweickert 1977), whereas goldfish (*Carassius auratus*) developed a tolerance to anesthetic (2-phenoxyethanol) over a period of 14 days and recovered faster upon repeated anesthetization (Weyl et al. 1996). In this study, control toadfish showed no difference in recovery of ventilation rate indicating that for non-invasive procedures, subsequent re-exposure is similar to the initial procedure. To allow implanted toadfish sufficient time to recover from the two initial anesthetic exposures, fish were not challenged again with AQUI-S 20E until the next day and only two fish retained the same units to allow re-testing. These toadfish showed delayed recovery in ventilation

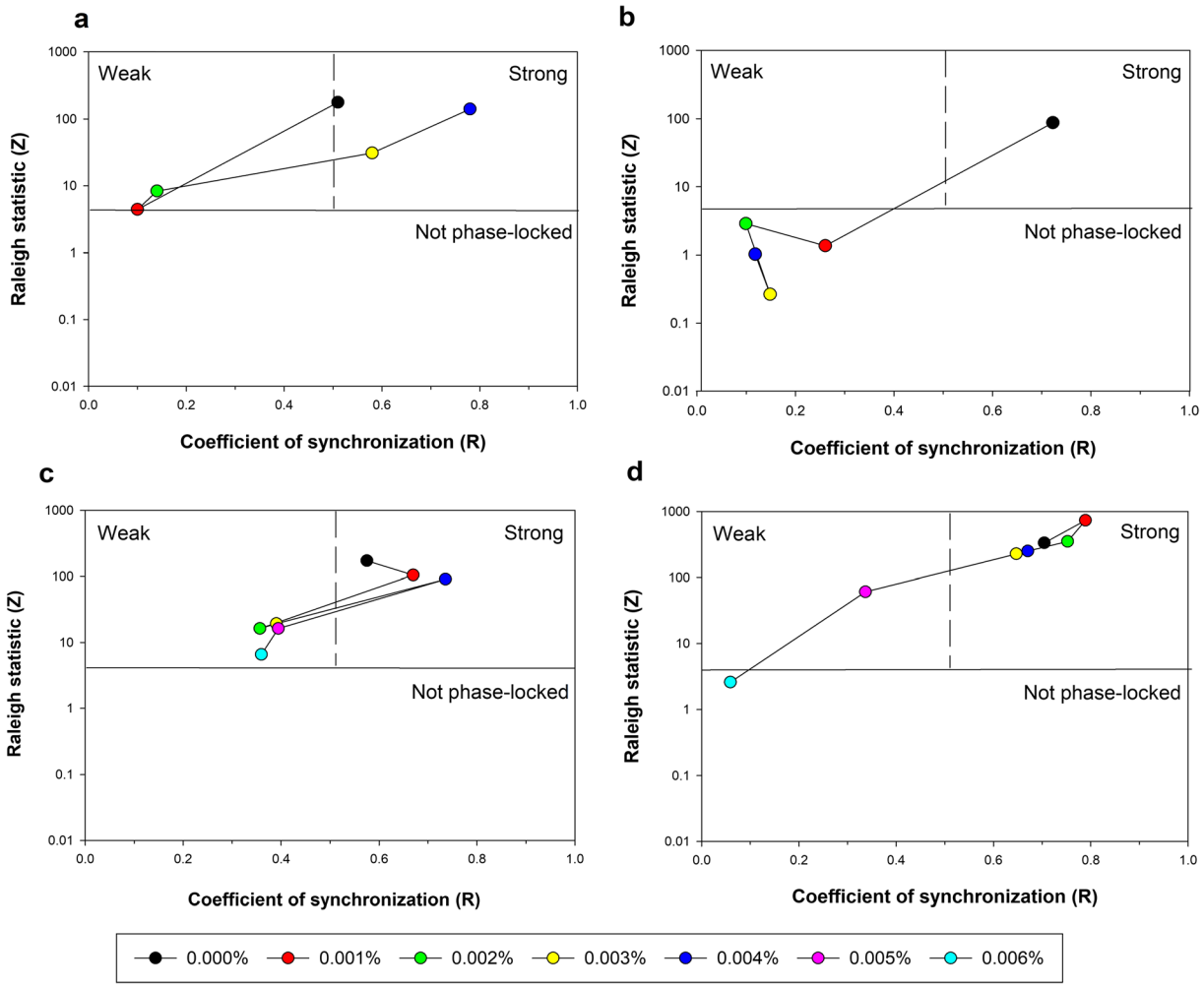

Fig. 5 The Rayleigh statistic plotted versus the coefficient of synchronization for 4 anterior lateral line fibers exposed to concentrations of AQUI-S 20E. The different colors show different AQUI-S 20E concentrations (0.000–0.0006%). **a** TF1 A; **b** TF4 A; **c** TF6 B; **d** TF7 A. The horizontal dashed line, $Z = 6.91$, distinguishes significantly phase-locked fibers, which are located above the line. The vertical dashed line at $R = 0.5$ divides strongly phase-locked fibers ($R > 0.5$) from weakly phase-locked fibers

rate; however, it is difficult to conclude whether this is attributable to anesthesia or cumulative stress from the previous day.

It has been suggested for short-term handling or invasive procedures such as implanting telemetry tags that anesthetic should have a short induction (< 3 min) and recovery time (< 10 min) with minimal hyperactivity or stress (Priborsky and Velisek 2018). However, AQUI-S 20E is an oil-based substance which coats the gill epithelia potentially blocking gaseous diffusion. A narrow safety margin was reported for lumpfish (*Cyclopterus lumpus*) anesthetized using AQUI-S 20E (0.0018%), with the average recovery time considered too long (15 min) and three out of ten fish not recovering (Jacobsen et al. 2019); and red pacu (*Piaractus brachypomus*) exposed to eugenol (0.0100%) required resuscitation to recover (Sladky et al. 2001). In this study, AQUI-S 20E was administered to unrestrained "free-swimming" toadfish and therefore the anesthetic was "self-induced" and dependent on the ventilation rate of the fish. The incremental exposure rates needed to establish the dose response curve precluded assessment of induction time; however, recovery (9.4 ± 6.4 min) in control fish was within the suggested time frame (Priborsky and Velisek 2018). While implanted fish had a longer recovery time (24.3 ± 16.8 min), all toadfish resumed normal swimming behavior and regained response to tactile stimulus within 60 min, [with no

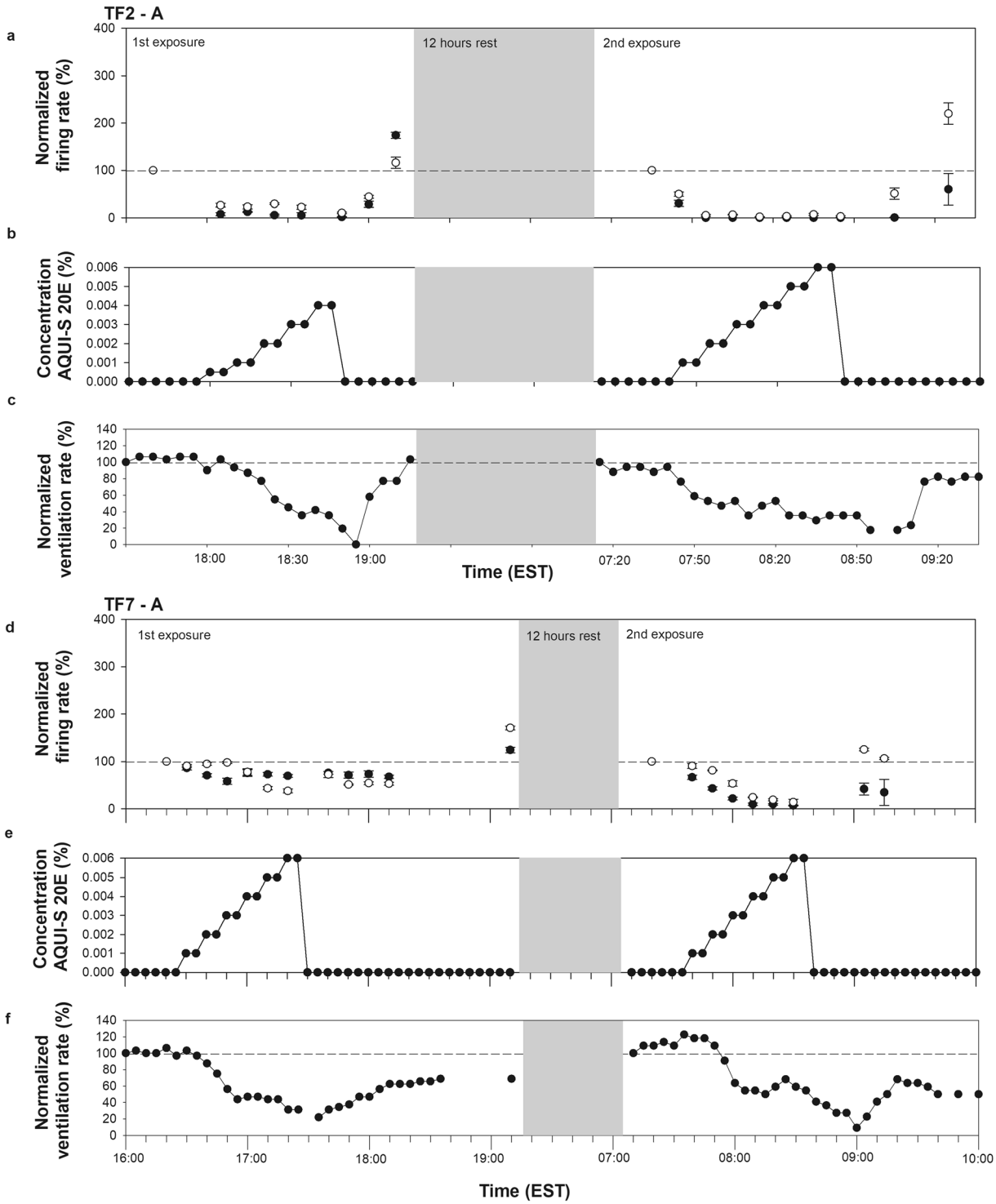

Fig. 6 Induction and recovery of lateral line fibers during repeated application and withdrawal of AQUI-S 20E anesthesia for two toadfish. **a**, **d** Spontaneous (filled circles) and evoked (open circles) neural activity in response to anesthetic concentration is plotted versus time (EST) during two exposures to AQUI-S 20E with a 12-h rest period in between. Firing rate is normalized as a percent of pre-anesthetized firing activity. Data is shown as mean ± 1 s.d. The dashed line represents the pre-anesthetized firing rate. **b**, **e** Ventilation rate of toadfish normalized as a percent of pre-anesthetized ventilation plotted versus time (EST). **c**, **f** AQUI-S 20E concentration of the saltwater bath immersion for the toadfish plotted versus time (EST). Note the different times for each fish

observable signs of hyperactivity or stress (Zahl et al. 2012)] indicating the safe use of AQUI-S 20E (< 0.006%) at least in this species. Furthermore, the kinetics of induction and recovery from this study should be considered conservative with faster times likely achieved with direct gill irrigation rather than self-induction.

Conclusion

This study investigated the neural response of the anterior lateral line when individual fish were exposed to AQUI-S 20E anesthetic. Increasing the concentration of AQUI-S 20E (0.001–0.006%) significantly decreased spontaneous and evoked firing rates of the anterior lateral line. The behavioral and neural effects of AQUI-S 20E were transitory as both spontaneous and evoked firing rates recovered in the majority of fibers (70%) within 30 min of anesthetic withdrawal. Ethical considerations surrounding invasive and noxious procedures present a dilemma for sensory physiologists because anesthesia influences neural activity of the targeted system. Although AQUI-S 20E does not appear to be a better option than MS-222 for neurophysiological studies, the depression of the nervous system may prove advantageous when shipping live fish in high densities or limited spaces and partially mitigate the stress response. This information will be helpful in developing standardized techniques for carrying out neurophysiology experiments, as well as the use for the aquaculture industry if this anesthetic agent is approved by the FDA in future years.

Acknowledgments The authors would like to acknowledge the Marine Resources Center staff at the Marine Biological Laboratory for providing toadfish care. We would also like to thank Lisa Abbo for veterinary advice on the project, and the Aquatic Animal Drug Approval Partnership Program (AADAP) and Bonnie Johnson at the (INAD) for overseeing animal care protocols when using AQUI-S 20E. The project was conducted under study #11-741-19-256.

Authors' contributions RLP, LSR, BG, and AFM: conception and design of research; RLP, LSR, and BG: performed experiments; RLP: analyzed data; RLP and LSR: interpreted results; RLP: prepared figures and drafted manuscript; RLP: wrote the first draft; LSR, BG, and AFM: edited, revised, and approved final version of the manuscript.

Funding Funding was provided by the Mistletoe Postdoctoral Fellowship to RLP, the National Science Foundation Graduate Research Fellowship Program under grant DGE 1762114 to LSR, and the National Science Foundation Grant IOS 1354745 to AFM.

Compliance with ethical standards

Conflict of interest The authors declare no that they have no competing interests.

Data availability All data for this study will be made available to the public in the Dryad repository following publication.

Code availability Not applicable.

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