The effects of high-intensity, low-frequency active sonar on rainbow trout

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This study investigated the effects on rainbow trout (*Oncorhynchus mykiss*) of exposure to high-intensity, low-frequency sonar using an element of the standard Surveillance Towed Array Sensor System Low Frequency Active (LFA) sonar source array. Effects of the LFA sonar on hearing were tested using auditory brainstem responses. Effects were also examined on inner ear morphology using scanning electron microscopy and on nonauditory tissues using general pathology and histopathology. Animals were exposed to a maximum received rms sound pressure level of 193 dB re 1 μPa^2 for 324 or 648 s, an exposure that is far in excess of any exposure a fish would normally encounter in the wild. The most significant effect was a 20-dB auditory threshold shift at 400 Hz. However, the results varied with different groups of trout, suggesting developmental and/or genetic impacts on how sound exposure affects hearing. There was no fish mortality during or after exposure. Sensory tissue of the inner ears did not show morphological damage even several days post–sound exposure. Similarly, gross- and histopathology observations demonstrated no effects on nonauditory tissues. [DOI: 10.1121/1.2735115]

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I. INTRODUCTION

There is growing concern among investigators, regulators, and the public that human-generated (anthropogenic) sounds in the marine environment could have deleterious impacts on aquatic organisms (e.g., National Research Council, 1994, 2000, 2003; Richardson *et al.*, 1995; Popper, 2003; Popper *et al.*, 2004; Wartzog *et al.*, 2004). However, only a few peer-reviewed studies address these concerns. Although much of the interest in the effects of marine anthropogenic sound has focused on marine mammals, concerns are now being raised about the effects of these sounds on fish (see Myrberg, 1980; Popper, 2003; Popper *et al.*, 2004, 2005; Hastings and Popper, 2005). Many of the physiological systems in fishes are similar to those in marine mammals, suggesting that environmental sounds could impact the survival and/or health and well-being of fishes as well as mammals.

The potential effects of high-intensity sounds on fishes (as on marine mammals—Richardson *et al.*, 1995) may include temporary threshold shift, increased stress levels (e.g., Hattingh and Petty, 1992), and/or damage to other organ systems including the circulatory system, neural tissue, etc. (e.g., Cernak *et al.*, 1996; Dodd *et al.*, 1997). There is also the potential for behavioral effects including movement away from the source and alterations in feeding and mating (e.g., Løkkeborg *et al.*, 1996; Engås and Løkkeborg, 2002). Although most sound exposures do not cause immediate

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death, as may occur as a consequence of exposure to the concussive forces of blasting (e.g., Yelverton *et al.*, 1975), the long-term effects of any of the stimuli may reduce the viability of animals or their reproductive success, with potential consequences at the population level.

Data on the direct effects of loud sounds on fishes are limited. Exposure to very loud sound for several hours results in damage to the sensory hair cells of the inner ear (Enger, 1981; Hastings *et al.*, 1996; McCauley *et al.*, 2003). Exposure to sounds for shorter periods can produce temporary hearing loss [i.e., temporary threshold shift] that lasts for some time after the termination of exposure (e.g., Popper and Clarke, 1976; Scholik and Yan, 2001; Amoser and Ladich, 2003; Smith *et al.*, 2004a, b; Popper *et al.*, 2005; Wysocki and Ladich, 2005). Lastly, almost nothing is known about the potential effects of sounds to other organ systems.

The current study examined whether exposure to high-intensity, low-frequency sonar, such as the Navy's Surveillance Towed Array Sensor System (SURTASS) Low Frequency Active (LFA) sonar, affects hearing sensitivity, the structure of the ear, and nonauditory systems in rainbow trout (*Oncorhynchus mykiss*). A LFA sonar array produces a signal with an effective source level of over 220 dB re 1 μPa^2 at 1 m and has the potential to ensonify fishes with sound pressure levels over 180 dB re 1 μPa^2 within 1 km from the ship-board array. Moreover, LFA sonar uses frequencies from 100 to 500 Hz, a range in which most fish are able to detect sound and the range of best hearing of many species (Fay, 1988; Fay and Popper, 1999; Popper *et al.*, 2003). Thus, LFA sonar not only has the potential to damage any number of organ systems in fishes due to the sound intensity but also has the direct potential of affecting hearing because the ears of fishes detect the operational frequency range of the sonar.

II. METHODS

A. Species

Species of great concern to users of LFA sound sources (as well as other human-generated sources) are listed and endangered salmonids on the West Coast of the U.S. While it would have been ideal to use those species in this study, their status precludes their availability for research. (Moreover, only species that are endemic to Seneca Lake could be used in this study.) At the same time, rainbow trout was available for these studies. This species has some potential for being a surrogate for a number of salmonids since it is of the same genus of most of the endangered and listed species. Furthermore, analysis of the structure of the ears of several different salmonid species, including rainbow trout (*Salmo gairdneri* Richardson), Atlantic salmon (*Salmo salar* Linnaeus) (Popper, 1977), Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) (Popper, 1977; Song, private communication, and other salmonids (Popper, 1976, 1977) show that the ear is very similar in all of these species. Moreover, the general structure of the auditory system, and the lack of specializations for enhanced hearing, is the same in all salmonids.

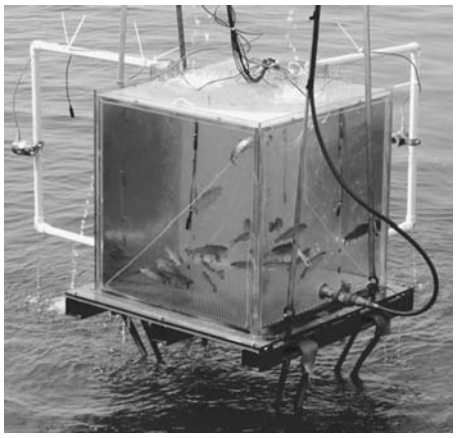

FIG. 1. The test tank with rainbow trout being removed from the water alongside the barge. The hose on the right brings cool water into the test tank. Hydrophones can be seen hanging inside the test tank and TV cameras were mounted on the two PVC brackets located on the outside. A 238.1-kg weight (below the water level) is suspended by slings from the bottom to stabilize deployment.

B. Test site

Experiments were conducted at the US Navy Sonar Test Facility in Seneca Lake, Dresden, NY (http://www.npt.nuwc.navy.mil/Seneca/index.html). The facility consists of a large barge in the center of the lake where the lake is 140.2 m deep. The facility includes a nearby shore support area with space for holding animals and for all post–sound exposure studies.

C. Test tank

Exposure to the LFA sound was conducted with fish in 1-m^3 test tanks made of 1.27-cm-thick clear Lexan® (Fig. 1) that were lowered into Seneca Lake from the barge (Fig. 2). Cool lake water was pumped into the test tank both during

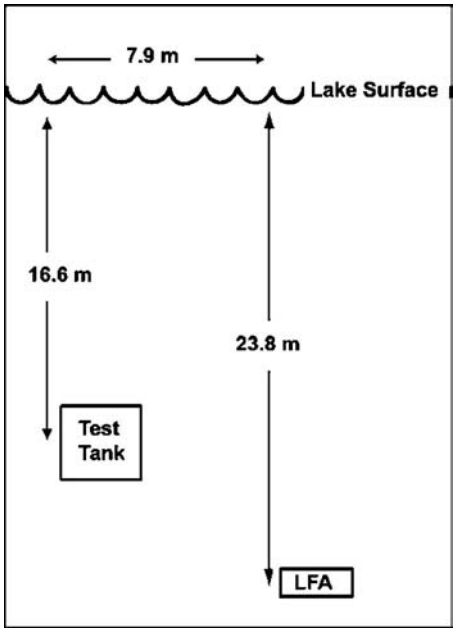

FIG. 2. Geometry of experimental setup. Distances are center of LFA-to-center of test tank and their respective depths. (Image is not to scale.)

experiments and on shore through an inlet valve located near the bottom of the test tank. Another valve placed near the top of the test tank allowed water to flow out. The test tank sat on a metal palette with 1.58-cm shoulder eyebolts that enabled the whole unit to be lifted by forklift or crane. The test tank was covered with a perforated polypropylene cover that allowed water to flow in and out of the test tank when it was submerged and for air to escape from within the test tank.

Six hydrophones (HighTech, Inc., model HTI-96-Min) were placed in the test tank during experiments. They were attached onto three fixed posts that were mounted to the perforated lid, with two hydrophones per post. Three of the hydrophones were 30.5 cm below the top of the tank and the other three were 20 cm lower. The output of each hydrophone was fed to a Wavebook (DAS model 516E) data-acquisition board using a sampling rate of 8 kHz and then to a Dell Inspiron 8500 laptop computer. An additional hydrophone was placed outside the test tank, 50.8 cm laterally to the test tank and 30.5 cm from the top. The hydrophone data-collection hardware was the same as for the calibration test (see the following). MATLAB was used to analyze the digitized signal for all experiments. Background noise spectral levels were measured daily during control tests and were consistently 100±2 dB re 1 μPa^2/Hz in the frequency band of interest.

Two Sea Viewer 650-420TVL video cameras were mounted on PVC brackets at right angles to each other on the outside of the tank (Fig. 1). These were used to monitor and record the behavior of the fish from the time the test tank entered the water until it was removed from the water. Data from the behavioral observations will be presented in a separate study.

D. Acoustic calibration in the test tank

During experiments and calibration, a crane lowered the test tank so that the center of the tank was 16.6 m below the surface of the water. A single LFA LTS (Low-Frequency Transmit System) transducer was at a depth of 23.8 m from its center to the water surface. The configuration of the setup is shown in Fig. 2.

Calibration of the system was performed to determine the maximum sound pressure level inside the submerged test tank and the uniformity of the sound field to provide a proper environment for the fish experiments.

Data from transmitted flat-spectra broadband noise (Fig. 3) in 1-Hz bins showed that the variation in level inside the test tank was ±3 dB. By comparison with the exterior hydrophone, it was also determined that the test tank was essentially acoustically transparent over the frequency range of interest. For a single tone, the maximum rms sound level was 193±3 dB re 1 μPa^2 at 196 Hz. Sound exposure level (SEL) is a measurement of a constant level of energy for 1 s. The calculated SEL was approximately 188.5 dB re 1 μPa^2 s. Finally, assuming particle velocity would be that for a propagating planar wave, the rms particle velocity is $v=p/\rho_0 c$, where p is rms pressure (~4500 Pa), ρ_0 is density (~1000 kg/m^3), and c is the speed of sound (~1500 m/s); thus the calculated maximum particle velocity inside the test tank was 0.003 m/s.

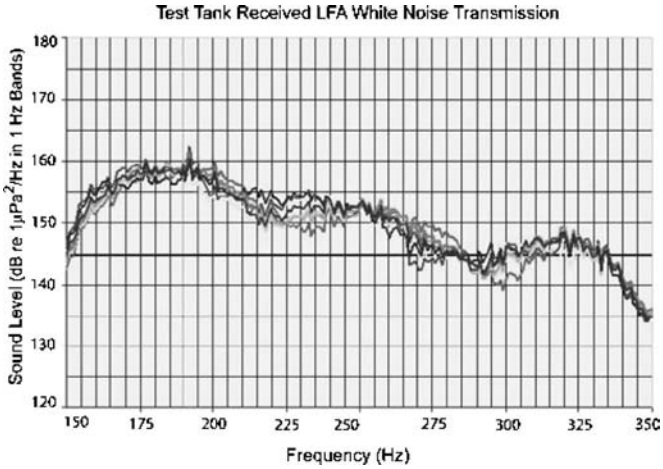

FIG. 3. White noise transmitted by the LFA as recorded on eight different hydrophones distributed throughout the TT.

E. LFA test sounds

Sounds were produced using the single LFA transducer driven by 1600 V by two s11-48 amplifiers (Instrument, Inc.) connected in series. The output of the transducer gave an approximate source level of 215 dB re 1 μPa^2 at 1 m. The signal used was generated via MATLAB and played out through the Dell Inspiron internal sound card. The signal was slightly modified from the actual classified sonar signal train used by the US Navy. The output of the transducer was constantly monitored to ensure the integrity of the test signal.

The frequency range of the signal was from 170 to 320 Hz (Fig. 4), and it consisted of three hyperbolic frequency-modulated (HFM) sweeps. Each hyperbolic sweep produces a curved output, spending more time on the lower

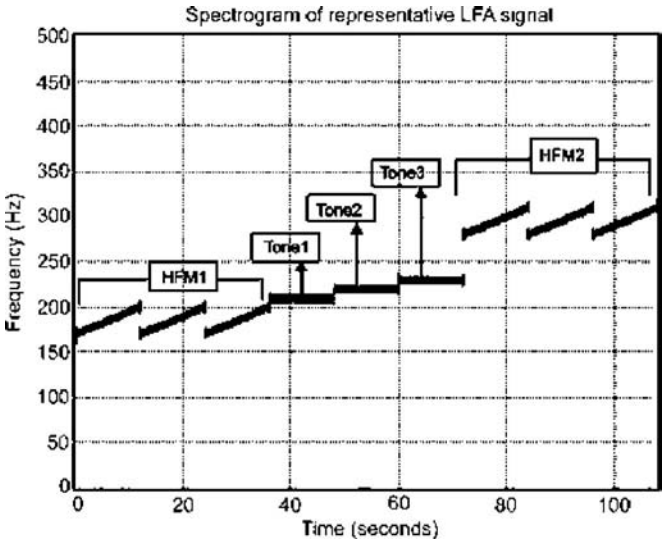

FIG. 4. Sound used in all experiments. The signal started with three HFM sweeps followed by three tones and concluded with three higher frequency HFM sweeps. (H=hyperbolic) (Note, in the MAX*2 experiments, this sequence was presented twice to give a continuous 216-s signal.)

TABLE I. Experimental groups defined.

Experimental groups	Definition
Baseline	Animals taken directly from the holding tank and not subject to any experimental handling. These served as "controls" for the actual control animals to ensure that handling and the experience in the experimental tanks did not cause any of the results ultimately found.
Control animals	Animals given the identical treatment as sound-exposed animals except that they were not presented with sound. They were kept at depth for the same length of time as the experimental animals.
MAX	Animals subject to three ensonifications of the signal (108 s duration) with a quiet period of 9 min between them.
MAX_24	Animals exposed to the maximum source level and tested 24 h postexposure.
MAX_48	Animals exposed to the maximum source level and tested 48 h postexposure.
MAX*2	Signal duration was twice that of MAX as was the off period. Source level was the same as in MAX.
MAX*2_24h	Animals exposed to MAX*2 and tested 24 h postexposure.
MAX*2_48	Animals exposed to MAX*2 and tested 48 h postexposure.
MAX-12	Animals exposed to MAX source level attenuated by 12 dB.
MAX-12_24	Animals exposed to the MAX source level attenuated by 12 dB; tested 24 h postexposure.
MAX-18	Animals exposed to MAX source level attenuated by 18 dB.

frequencies. This type of signal is a re-creation of the way the Navy transmits the signal when it uses the LFA sonar. The first part of the signal was a tone swept from 170 to 210 Hz three times followed by three tones at 210, 220, and 230 Hz, finally followed by three more HFM sweeps from 280 to 310 Hz.

Tests were conducted with several different sound exposures as described in Table I. Test tones in all but the MAX*2 experiments consisted of three repetitions of test sound (108 s each) separated by a 9-min silent period. In the MAX*2 trials, each sound presentation was repeated twice in succession for a 216-s-long signal with an 18-min quiet period. The longer quiet interval was required with MAX*2 in order to maintain a 16.7% duty cycle for all experiments and to allow the LFA transducer to cool. The overall sequence for each test was lower test tank to depth-transmit the signal-quiet-repeat signal-quiet-repeat signal-lift test tank out of the water onto barge.

F. Fish handling

Fish (170–275 mm total length; 90–212 g) were obtained from the Fingerlakes Fish Farm, Seneca Falls, NY. The history of the fish, including the source of eggs, could not be ascertained. Prior to transport to the Seneca Lake test facility, fish were gradually temperature acclimated to test conditions. They arrived at the study site in good health. Upon delivery, fish were transferred to holding tanks into which fresh, cool lake water was continuously circulated to maintain water quality. All fish handling and experiments were supervised and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, College Park, MD.

Prior to each experiment, and at least 12 h before being used in an actual experiment, 20 fish were transferred from the holding tanks into one of three test tanks. Until the test tank was actually submerged for tests, it was enclosed in insulating foam to help maintain water temperature and to prevent the fish from receiving external visual stimuli, thereby minimizing stress in response to movements of the investigators.

When a test tank was to be used for a trial, it was lifted by a crane, placed onto a workboat, and transferred to the barge. Noise spectral levels within the test tank during transport to the barge were measured as 105 ± 5 dB re 1 $\mu Pa^2/Hz$. Once on the barge, the insulation was removed and the test tank was fitted with the perforated cover that included the hydrophones and video cameras (Fig. 1). The test tank was again hooked to a flow-through water system to maintain aeration and a stable temperature for the fish.

Setup of the test tank on the barge, which involved putting hydrophones and cameras in place and hooking to the crane for lowering into the water, took approximately 10 min. It was then lifted by a crane and slowly lowered to the required depth over 15 min to allow the fish to acclimate to the change in water pressure. After the sound presentation, the test tank was lifted to the surface over 15 min and placed on the barge, the electronics were removed, and the insula-

tion was replaced. The test tank was then placed on a workboat and returned to shore where it was again hooked up to the lake water flow-through system.

G. Determination of hearing sensitivity

Once the test tank was returned to shore, three to six fish were removed sequentially for hearing tests at the US Navy shore facility on Seneca Lake. Once hearing tests were completed, fish were prepared for analysis of ear structure (see the following). Additional fish from each tank were used for pathology (see the following). The remaining fish were transferred to holding pens and tested up to 48 h postexposure to determine if there were changes in hearing capabilities and/or tissue damage. Classification of experimental groups follows the notation in Table I.

Hearing capabilities were measured using auditory brainstem response (ABR), a noninvasive method (e.g., Corwin *et al.*, 1982; Kenyon *et al.*, 1998). The ABR tank is a 35.6-cm-deep steel cylinder, 35.6 cm in diameter, with 1.27-cm-thick walls. An underwater speaker (UW30) is mounted through the bottom of the ABR tank. The tank has three legs 50.8 cm long, each with a vibration-isolation damping foot.

Fish were lightly anesthetized with buffered MS-222 and restrained in a mesh sling where the fish were suspended so that the top of the head was approximately 13 cm below the surface of the water.

A stainless steel electrode (Rochester Electro-Medical, Inc.) was inserted subdermally into the medial dorsal surface of the head between the nares as a reference electrode, while another electrode was inserted subdermally into the dorsal midline surface of the fish directly over the brainstem to record the brainstem neural response to sound stimulation. A ground electrode was placed in the water. All exposed metal surfaces of the reference and recording electrode tips that were not below the fish skin were insulated with cured fingernail polish.

Signals and the levels were produced and calibrated using a Tucker-Davis-Technologies (TDT) System 3 physiology apparatus with SigGen and BioSig software (TDT). The signals were played out through TDT RP2.1 and passed through a power amplifier (Hafler P1000) connected to the underwater speaker. Presented signals were 100-, 200-, and 400-Hz tone bursts with a 2-ms rise and fall time, 10 ms in duration, and were gated through a Hanning window. Brainstem responses were collected using the BIOSIG software package with at least 200 responses averaged for each presentation. The resulting levels of each presented frequency were confirmed using a hydrophone (G.R.A.S. 10CT hydrophone with a receiving sensitivity of -211 dB ± 3 dB re 1 V/μPa connected to a Kistler 5010 dual-mode amplifier).

The signal levels at each frequency were changed in 5-dB steps until a typical ABR wave form could not be seen above the noise. Threshold is defined as the lowest intensity level where a response above the background noise could be seen. The traditional determination of threshold is achieved through visual inspection, which provides results that are virtually identical to those determined using statistical approaches (Mann *et al.*, 2001; Higgs *et al.*, 2003). In addition, two experimenters independently evaluated all data and found agreement with the determined thresholds.

The focus of these hearing experiments was on relative measures of hearing sensitivity (Hawkins and Johnstone, 1978), i.e., *changes* in hearing sensitivity as a result of exposure to the LFA source. Thus, data are provided as relative measures of threshold in terms of differences between experimental and control hearing thresholds. Relative measures were maintained by placing fish at the same depth and position in the ABR tank and by keeping the water depth the same for all experiments.

H. Statistical analysis of hearing tests

All data sets were tested for normal distribution using the Kolmogorov-Smirnov test. When data were normally distributed and showed homogeneous variances, audiograms of the different exposure groups were compared using a two-way analysis of variance (ANOVA) following a general linear model where one factor was treatment (exposure group) and the other factor was frequency (100, 200, and 400 Hz). The treatment factor alone indicates overall differences between exposed groups of animals. When the treatment factor is combined with the frequency factor, it indicates overall differences at the tested frequencies.

In order to determine which frequencies differed in their thresholds, one-way ANOVAs were followed by Scheffé's multiple comparison procedure at each test frequency. The p values of statistical significance for these one-way ANOVAs and Scheffé's were adjusted (using the Bonferroni correction) by 3, for the number of frequencies tested ($^{*}p \leqslant 0.0167$, $^{**}p \leqslant 0.0033$, $^{***}p \leqslant 0.0003$).

For nonparametrical data, Kruskal-Wallis tests were used at each frequency to test overall group differences, followed by pairwise Mann-Whitney U tests. Levels of significance were adjusted using the Bonferroni correction, as above.

I. Necropsy and histopathology

Fish from the first two experimental weeks (Group I—see the following) for Controls ($n=30$), MAX ($n=22$), MAX*2 ($n=19$), and Baseline ($n=6$) were sacrificed using buffered MS-222. Gross pathological observations and length and weight data were taken for each fish after sacrifice (Kane, 1996; Kane *et al.*, 2000). Gross pathological observations examined the condition of the skin, eyes, fins, mesenteric vasculature, and the swim bladder to assess the general condition of the fish and look for possible exposure-related hemorrhage. General parasitology was conducted from skin scrapes and gill biopsies and ranked based on incidence of the different taxa observed.

Blood samples were drawn from the caudal vein, transferred to heparinized hematocrit tubes, sealed, and centrifuged to determine hematocrit (packed red blood cell volume). Hematocrit tubes were also observed for sanguineous color in the plasma layer that might be indicative of possible LFA effect on red blood cells.

TABLE II. Auditory threshold differences between postexposure means and controls (combined baseline and control) means at each test frequency (mean dB ± SEM).

	Δ 100 Hz	*n*	Δ 200 Hz	*n*	Δ 400 Hz	*n*
			Group I. May and June, 2004			
MAX	4±1.95	16	5±1.83	17	21±2.20	20
MAX_24h	2±1.63	9	4±1.57	10	25±2.33	10
MAX*2	−3±1.42	6	−2±1.26	6	20±1.50	6
MAX*2_24h	0±1.73	4	−3±1.73	4	17±1.73	4
MAX*2_48h	0±1.94	4	1±1.88	4	25±1.63	4
			Group II. October, 2004 and May, 2005			
MAX	−2±2.34	12	−1±1.91	12	3±2.37	12
MAX_24h	−2±2.29	12	−2±2.30	12	1±2.59	12
MAX_48h	2±3.19	6	−2±3.20	6	−5±3.26	6
MAX-12	4±2.09	18	5±2.07	18	5±2.08	18
MAX-12_24h	−3±2.36	12	−3±2.32	12	3±2.42	12
MAX-18	0±2.64	10	−3±2.80	10	5±2.61	10

Histopathology samples ($n=8-10$ per exposure group) were taken from necropsied specimens immediately after sacrifice and external examination (Kane, 1996). During necropsy, the swim bladder was examined for any signs of hemorrhage that might be associated with sound exposure. The presence of any external or internal anomalies were noted and digitally photographed. Tissues, including eye, skin, gill, muscle, heart, posterior kidney, liver, spleen, and swim bladder, were preserved in 10% neutral-buffered formalin. Preserved tissues were processed for routine histology and stained with hematoxylin and eosin prior to coverslipping.

Glass slides were observed blindly (without knowing the exposure group) to evaluate the various tissues for histopathology. Tissue-level anomalies were noted and ranked on a scale of 0–5, where 0=no pathology and 5=severe. Examples of lesions typical of an exposure group, if any, were digitally photographed and recorded.

J. Examination of inner ear tissue

Immediately after ABR, each fish was euthanized with buffered MS-222. Some fish ear tissue was obtained 24, 48, and 96 h postexposure. The cranium was opened, and fixative (2.5% gluteraldehyde and 2.5% paraformaldehyde in 0.1 Millonig's buffer at pH 7.4) was injected into the cranial cavity as the initial fixation. The cranium and the ear were then placed in cold fixative solution for at least 24 h and then transferred to cold buffer for storage until use.

The ears were then dissected from the cranium, counterstained with 1% osmium tetroxide, and dehydrated through a series of ethanols to 70%. The sensory epithelia were then removed for SEM, dehydrated in 100% ethanol, and critical point dried using liquid carbon dioxide as the intermediary fluid. After mounting on aluminum stubs, the epithelia were coated with a 25-nm-thick layer of platinum and studied using an AMRAY model 1820 scanning electron microscope.

The sensory cells of the three otolithic end organ epithelia (saccule, lagena, and utricle) were examined for possible morphological damage resulting from LFA exposure. Tissue was examined in terms of the integrity of the sensory tissues by comparing exposure groups to control and baseline animals. In addition, the results were compared to morphological hair cell damage that was encountered in a number of earlier studies (e.g., Enger, 1981; Hastings *et al.*, 1996; McCauley *et al.*, 2003).

K. Grouping experimental weeks

The results reported here were determined during four different experimental weeks at Seneca Lake—May, June, and October, 2004 and May, 2005. The hearing data for May and June, 2004 were similar and they are pooled in the following discussions. Similarly, the hearing data for October, 2004 and May, 2005 were similar and are pooled. For ease of discussion and because differences existed in each group's respective baseline thresholds, May-June (Group I) and October-May (Group II) data are reported separately. The difference between the combined control/baseline thresholds of Group II from Group I was 20 dB at 100 Hz, 12 dB at 200 Hz, and −18 dB at 400 Hz (i.e., Group I control thresholds were 20 dB greater than Group II, etc.). Mean data for the two groups are presented in Table II. Nonparametrical statistical tests were applied to Group I results, whereas parametrical tests were used for Group II.

III. RESULTS

A. Baseline and control hearing thresholds

In order to determine whether there were threshold shifts due to LFA exposure or due to the handling of the fish, effects on hearing and on body tissues were measured in control and baseline animals. Controls were treated precisely as exposed animals other than for actual ensonification. Baseline animals were from the same stock as control and exposed animals but they were not placed in test tanks or transported to the barge. The baseline served as a control for handling. There were no differences in the detection sensitivity between control and baseline for either group (Group I: Mann-Whitney U tests at each frequency, all p values >0.05; Group II: unpaired t-tests at each frequency, all p values >0.05). Consequently, data for the baseline and control animals within each group were pooled.

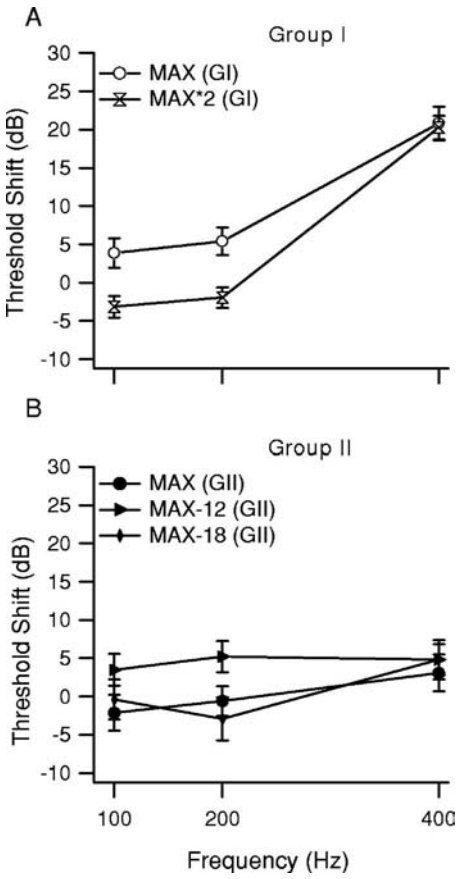

FIG. 5. Relative thresholds shifts immediately after LFA exposure; (A) Group I (GI) includes MAX and MAX*2 exposures; significant shifts were at 400 Hz for both exposures. (B) Group II (GII) includes MAX, MAX-12, and MAX-18 exposures. A significant shift was at 200 Hz for MAX-12. (Note: Auditory thresholds after exposure were subtracted from the combined baseline/control auditory thresholds giving threshold shifts.)

B. Hearing thresholds immediately after exposure to LFA sounds

Kruskal-Wallis tests at each frequency (levels of significance had Bonferroni correction) in Group I animals revealed significant differences in the ABR thresholds between control, MAX and MAX*2 animals at 200 Hz [Fig. 5(a)] ($\chi^2_{2,41}=8.62$, $p=0.013$) and at 400 Hz ($\chi^2_{2,43}=27.56$, $p=\leqslant 0.001$).

At 400 Hz, the thresholds of MAX and MAX*2 animals were 20 dB higher than those of the control animals and significantly differed from the control values ($p\leqslant 0.001$) and not from each other ($p=0.322$). The difference at 200 Hz was mainly due to the MAX group having an average threshold 5 dB higher than that of controls [Fig. 5(a), Table II]; however, subsequent Mann-Whitney *U* tests did not reveal this to be significantly different.

Comparisons between audiograms of Group II animals showed significant overall differences between the exposure groups (two-way ANOVA: $F_{3,191}=7.14$, $p\leqslant 0.001$) but no significant interaction between treatment (exposure groups) and frequency ($F_{6,191}=1.64$, $p=0.138$), indicating that the changes in thresholds showed the same trend at all frequencies tested. This difference was due to the MAX-12 animals being significantly different from all the other groups (Scheffé's post hoc tests: $p<0.05$ in all cases). Subsequent one-way ANOVAs at the separate frequencies showed that this difference was due to a shift in thresholds at 200 Hz ($F_{3,63}=5.61$, $p=0.002$), (Fig. 5).

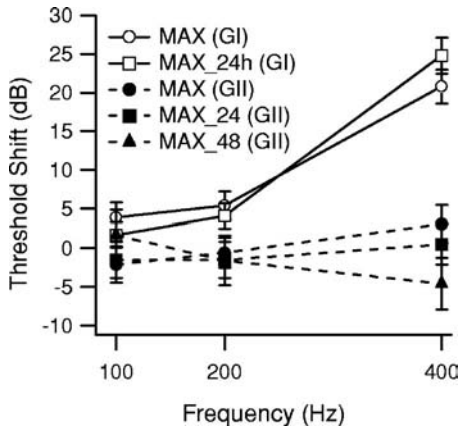

FIG. 6. Postexposure threshold shift for Group I (GI) and Group II (GII) trout following MAX exposure. Group I (open symbols) showed significant threshold shift at 400 Hz out to 24 h. Group II (closed symbols) did not show threshold shifts and there was no threshold change up to 48 h postexposure.

C. Postexposure hearing thresholds

In order to determine the time course of threshold shift after exposure, rainbow trout were tested up to 48 h postexposure. Group I showed significant threshold shifts in postexposure auditory measurements with threshold shifts at 400 Hz of 25 dB for MAX_24h, 17 dB for MAX*2_24h, and 25 dB for MAX*2_48h (Table II). Controls, MAX, and MAX_24h animals showed significant differences at 400 Hz (Kruskal-Wallis: $\chi^2_{2,47}=30.26$, $p\leqslant 0.001$) and none at 100 or 200 Hz. At 400 Hz for both MAX and MAX_24h, thresholds significantly differed from control thresholds (Mann-Whitney *U* tests: $p\leqslant 0.001$ in both cases): indicating significant threshold shifts remained 24 h post-LFA exposure (Fig. 6).

Control, MAX*2, MAX*2_24h, and MAX*2_48h animals showed significant differences at 400 Hz (Kruskal-Wallis: $\chi^2_{3,32}=25.87$, $p\leqslant 0.001$) and none at 100 or 200 Hz. Subsequent Mann-Whitney *U* tests showed that MAX*2 as well as MAX*2_24h and MAX*2_48h were significantly different from control thresholds ($p\leqslant 0.001$ in all cases), indicating that auditory thresholds at 400 Hz were still elevated relative to controls even 48 h postexposure (Fig. 7).

In Group II, the auditory thresholds of MAX animals were not different from the controls. A two-way ANOVA (including controls, MAX, MAX_24h, and MAX_48h) showed no difference in thresholds out to 48 h postexposure ($F_{3,161}=0.52$, $p=0.671$). In contrast, the control and MAX-12 auditory thresholds differed significantly (two-way ANOVA: $F_{2,161}=12.33$, $p\leqslant 0.001$ for the factor treatment group). MAX-12 differed significantly from the control values ($p<0.001$).

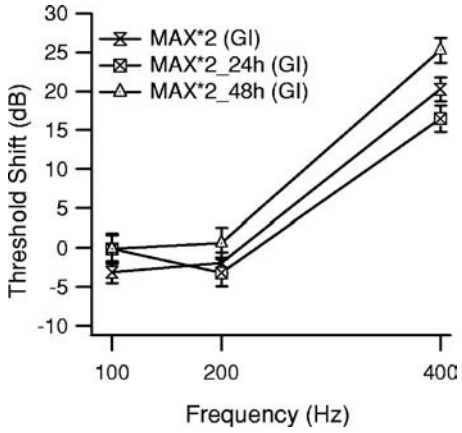

FIG. 7. Post-MAX*2 exposure threshold shifts for Group I trout. Significant shifts were at 400 Hz for all three test groups.

D. Effects on inner ear morphology

The morphology of the sensory epithelia of the end organs of LFA-exposed fish were examined and compared to control and baseline fish. Figure 8 shows the results from MAX exposure, while Fig. 9 shows MAX*2 exposure. There was no apparent morphological damage to any end organ sensory epithelia or sensory cells in any of the exposure groups, even in fish that were examined 96-h postexposure. (Note, 96 h animals were not tested for hearing sensitivity due to time constraints during the weeks at Seneca Lake.)

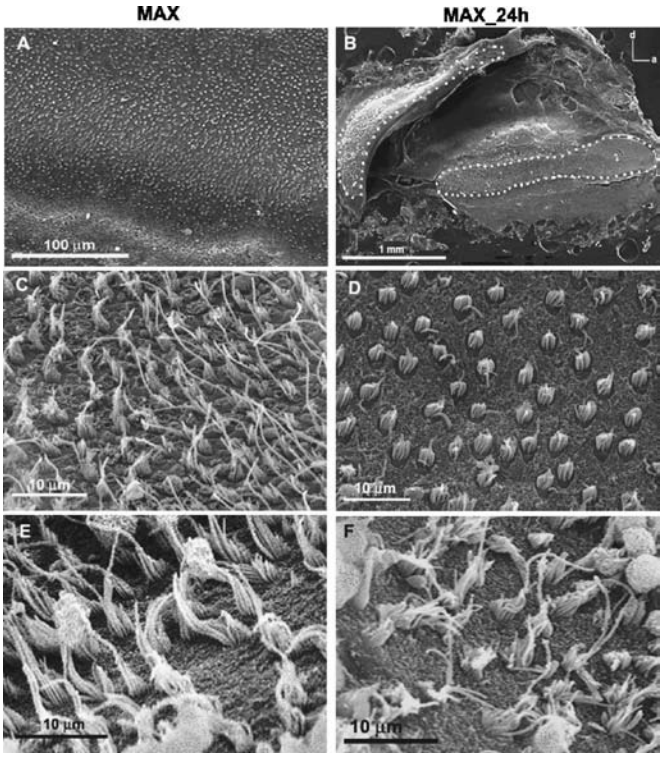

FIG. 8. SEM of auditory sensory epithelia and hair cells from Group I trout. (A) MAX exposure: Low-power image of a region of the left saccule. Note that there were no missing ciliary bundles. (B) MAX_24h: Low-power image of a complete lagena (left) and complete saccule (right). The sensory epithelia are outlined with a dashed line. Both end organs were completely intact, and there was no indication at low power of damage. (C) MAX exposure: High-power image of ciliary bundles on a region of the right utricle. (D) MAX_24h: A region of the left saccule. There was no indication of damage. Note the ciliary bundles on the saccule are shorter than those on the utricle in C. (E) MAX exposure: A region of the left lagena. (F) MAX_24h: A region of the left lagena.

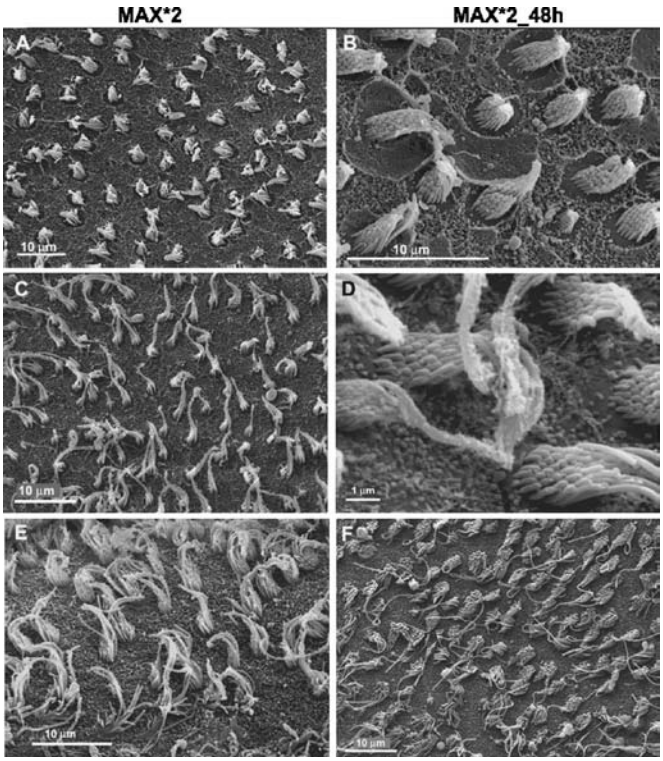

FIG. 9. SEM of auditory sensory epithelia and sensory hair cells of Group I. (A) MAX*2 exposure: Low-power image of the right saccule. (B) MAX*2_48h: left saccule; (C) MAX*2: right utricle; (D) MAX*2_48h: right utricle at higher power to show details of the stereocilia and how they are graded in size. (E) MAX*2: left lagena. (F) MAX*2_48h: left lagena.

E. Gross pathology and histopathology

Eighty-two rainbow trout were examined (26 controls; 56 exposed) from Group I experiments for general observations and gross pathological changes associated with exposure to LFA, while eight animals from each sound exposure parameter were analyzed for histopathology. All comparisons were between exposed animals versus baseline and control animals. Several animals exhibited mild to moderate skin or fin abrasion but these alterations were likely due to transport and/or netting since similar abrasions were found in baseline, control, and exposed animals. There were no changes to the external anatomy of any of the sound-exposed specimens and no observations of bleeding from the swim bladder or any vasculature. Swim bladders from all groups (exposed, baseline, and control) were intact and still inflated upon necropsy.

Blood was taken from baseline, control, and exposed fish to observe the plasma for the presence of pink or red coloration as a sign of possible erythrocyte membrane disruption in association with LFA exposure. Hematocrit data were derived from two control groups ($n=15$), two MAX groups ($n=20$), and two MAX*2 groups ($n=16$). The mean hematocrit values for the control, MAX, and MAX*2 groups were 43.6%, 43.5%, and 44.4%, respectively. A parametric ANOVA was used to analyze hematocrit between exposed

groups because the data met the assumptions of normality and homoscedasticity. There were no statistical differences between the hematocrits of these groups ($p=0.92$) and the hematocrit values derived were within normal range for rainbow trout (Rehulka and Minarik, 2004). The plasma of all of the blood samples was clear to straw colored and was not pink or red, indicating that erythrocytes remained largely intact throughout the different exposures as well as the blood collection.

Parasitology data from rainbow trout gill biopsies and skin scrapes indicated the presence of several different taxa of external parasites. There were three protozoan ciliate parasites observed (*Trichodina, Ichthyopthirius*, and *Ambiphyra*), and one flagellate (*Ichthyobodo*). Metazoan parasites included monogenean trematodes (skin and gills) and copepods (gills only). Ranking of parasite observations ranged from 0 to 3 for any given parasite. For statistical analysis, each exposure group from the different experimental replicates (control, MAX, and MAX*2) were tested for differences using nonparametric Kruskal-Wallis. No significant differences were found among the exposure groups; therefore, the data for each exposure were pooled for analysis. A Kruskal-Wallis ANOVA was used to test for differences between exposure groups (control, MAX, and MAX*2) and parasite rank. There were no statistical or empirically observed differences in parasite rankings between control and LFA-exposed groups, indicating that acoustic exposure did not alter the incidence of parasite observations.

Observations were made on histological tissues from brain, eye, heart, gills, stomach, intestine, liver, pancreas, spleen, anterior kidney, posterior kidney, swim bladder, skin, muscle, and gonad. There were no exposure-related pathologies observed in any of the tissues from exposed or control animals (Fig. 10).

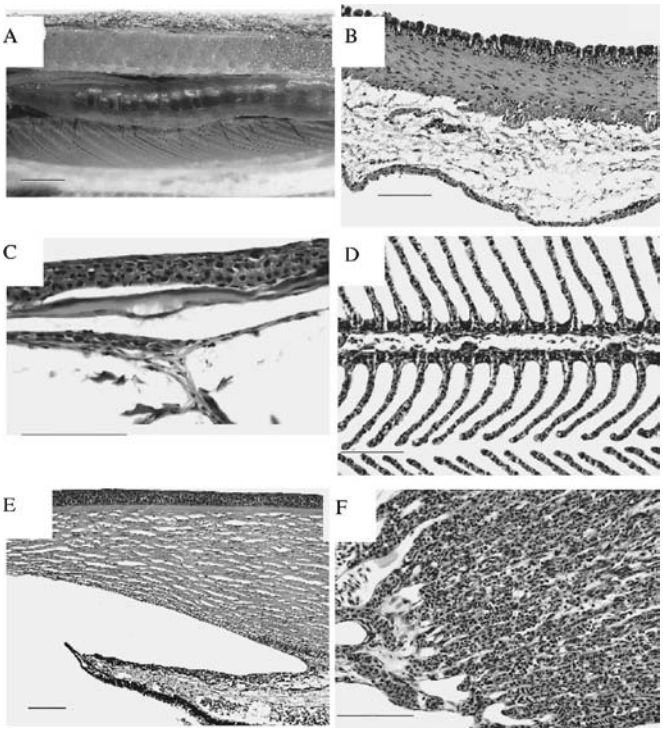

FIG. 10. Typical gross and histological observations from MAX-exposed rainbow trout. Observations of various tissues focused on different epithelia as well as areas of microvascularization. (A) Grossly intact, inflated swim bladder after removal of visceral organs (scale bar 10 mm). (B) Cross section of swim bladder (scale bar 100 μm). (C) Cross section of skin showing normal epithelium and scale pocket (scale bar 100 μm). (D) Gill tissue showing primary and secondary lamellae with intact epithelium and supporting cells (scale bar 100 μm). (E) Cross section of eye showing corneal epithelium and iris. (F) chorid (rete) of the eye (scale bar 100 μm).

IV. CONCLUSIONS

The order of conclusions begins with the experimental paradigm this study generated and followed, which serves as a guideline for future research studying the effects of noise exposure on fish. Next is a discussion of results of hearing sensitivity and the differences between Group I and Group II, followed by histopathology results. Finally, there is a discussion of the effects of LFA sonar on fish and the application of the results reported here to other species and other sources of intense sounds.

A. Guidelines for noise exposure studies

The Naval Sonar Test Facility (the experimental site) enabled development of an experimental paradigm that is optimal in terms of acoustic environment, calibration of the sounds to which the fish were exposed, and the ability to use a sound source that is virtually the same intensity as under actual use. In essence, this study was designed to eliminate the problems in earlier studies that resulted from lack of ability to calibrate the sound field and have control over most experimental parameters. The study provides a general experimental approach applicable to experiments on the effects of other anthropogenic sounds. Of course, the facilities available for this study were exceptionally conducive to this kind of work and access to comparable facilities for studies of other types of sounds may be limited. Thus, while future studies may not be able to precisely replicate the procedures used here vis-à-vis sound presentation, there are several experimental "principles" developed in this study that are applicable to other investigations. These include the following.

(1) Use of a fully calibrated and consistently monitored sound source. To characterize the sound field, pressure measurements were simultaneously made at six locations. The measured pressure was within 3 dB at all locations, indicating little pressure gradients. This is consistent with expectations of being in the free field and justifies an assumption that the particle velocity is related to the pressure by $(1/\rho c)$. Thus, signal characteristics were known and compared between trials, allowing for assurance that the signal was consistent.
(2) The test tanks were thoroughly analyzed in terms of acoustics (i.e., sound pressure, particle velocity, uniformity of signal inside the test tank, signal itself, etc). Thus, all factors of the sound field to which the fish were exposed were known.
(3) There was constant monitoring and digital storage of LFA signal presentations inside the test tank during all experiments. The sound pressure levels, frequency, envelope of the signal, and other sound characteristics were analyzed after each presentation.

(4) Each experiment, from the moment the test tank containing fish was placed in the water to the time it came out was video recorded and digitally stored. This provided a full record of the experiments for later analysis and allowed for detailed behavioral analyses to determine how fish responded to the sound.
(5) Relative hearing studies were done on fish immediately after sound exposure and continued for several days postexposure to investigate delayed effects and/or potential recovery.
(6) At least two ABR experts independently examined the hearing data sets. Thus, there was no bias in determining hearing thresholds based upon any preconceived expectations. The nature of the experiments did not allow for a double-blind study because the results from each day influenced the subsequent test days within each experimental week.
(7) Morphological analysis of the inner ear epithelia and sensory hair cells was performed on test animals by an expert anatomist.
(8) Gross pathology and follow-up histopathology were done by an expert fish pathologist. Internal organs, including the swim bladder, and associated microvasculature of exposed fish were examined and compared with control and baseline animals.
(9) The studies were done in a realistic environment that provided an excellent sound field as well as control of most variables that typically impact a "field" study.
(10) A significant number of control tests were performed along with an additional group of controls (referred to as baseline) for handling of the experimental and control animals.

B. Effects on hearing sensitivity

This is the first study to examine the effects of high-intensity low-frequency sonar on any fish species and is one of only two published studies to examine effects of any high-intensity sound on fish hearing (Popper *et al.*, 2005). The results from the hearing tests using ABR demonstrate that exposure to the LFA may affect hearing thresholds. However, as discussed in the following, the exposure to the LFA signal paradigm used in this study is more severe than fish would encounter in the wild. Therefore, the hearing losses found here represent an absolute worst-case scenario for hearing exposure.

There is evidence for threshold shifts in Group I at 400 Hz in rainbow trout lasting 48 h postexposure. In Group I at 400 Hz, the MAX and MAX_24h exposures had 20–25 dB shifts and the MAX*2, MAX*2_24h, and MAX*2_48h had shifts ranging from 17 to 25 dB (Table II). However, it should be noted that limited numbers ($n=4$) of the MAX*2_24h and MAX*2_48h were available and thus may not offer sufficient statistical power. Regardless, the overall results from Group I show that LFA sonar exposure does affect trout hearing thresholds and may last for at least two days.

In Group II, the only significant threshold shifts occurred at MAX-12 exposure. The fact that the maximum exposure (MAX) did not cause threshold shifts is perplexing. The Group II control fish had a 20-dB lower threshold at 200 Hz than the Group I fish, which could account for the shift that occurred at 200 Hz but does not offer any explanations as to why the MAX exposures had no effect on hearing thresholds. Also perplexing was the finding that Group I MAX had a large threshold shift at 400 Hz and Group II MAX had no change in threshold sensitivity. But, for Group I control fish-thresholds at 400 Hz were 20 dB lower than Group II control. This could somewhat account for the lack of an effect seen in Group II MAX at 400 Hz. Overall, Group II's data supports Group I's results that the LFA does affect hearing sensitivity, but the results from Group II call into question the degree to which the very long exposure to LFA signals affects fish hearing sensitivity.

It is possible that a 10–20 dB threshold shift at one frequency has an impact on survival of fish due to a decreased ability to detect biologically relevant sounds. However, not enough is known about the use of sounds by rainbow trout (or any other species) to determine whether such a decrement in hearing at a limited point of its hearing bandwidth would impair the detection of relevant sounds and the consequences of this for fish survival.

C. Differences in exposed groups

An issue with this study was the substantial differences in thresholds between Group I (May/June, 2004) experiments and Group II (October, 2004/May, 2005) experiments. The basis for these differences is not immediately clear. All animals came from the same supplier and were maintained and handled in an identical manner once they were brought to the experimental site, and the actual experiments were identical during each test week.

Factors that could not be controlled were the way the fish were raised at the fish hatchery and the genetic stock from which the animals came (something not controllable by fish farms). While all fish used in this study were of similar size, it is possible that their husbandry treatment had differing conditions at various times of the year or factors such as egg manipulation, water quality, feed, or other variables that could not be controlled. How such factors might affect hearing is unknown and would be almost impossible to control for.

Recent studies have shown that a hatchery-raised salmonid may differ in hearing sensitivity depending upon whether their otoliths (ear stones) have the typical aragonite crystal structure or if the otoliths are the far less common (and perhaps abnormal) vaterite crystalline form of calcium carbonate (Oxman, private communication). The discovery of these structural differences appears to be related to the way the fish were raised in hatcheries or to genetic diversities. Thus, if Groups I and II, used in the LFA study, were raised in dissimilar ways, it is possible that they had varying otolith structures and this could, in part, explain the different results reported here.

Additionally, a recent study on hearing sensitivity of two cohorts of rainbow trout from the same eggs but with slightly different handling prior to hatching showed significant dif-

ferences in hearing sensitivity, and this difference continued to show up over nine months of repeated testing (Wysocki, private communication). It is certainly the case that the LFA fish from Groups I and II came from different stocks, and it is possible that there are some genetic factors which impact hearing sensitivity and/or sensitivity to potential effects on hearing by anthropogenic sound.

The differences between Group I and II results are of interest and concern, and it may be key in demonstrating that reaching conclusions about the effects of sound on fish is not as simple as calculating sound levels or examining a single species. There is variability among fish within a species and going a step further to compare one species to another species becomes even more laden with complexities. These results may point to potential variability in effects based on a broad range of physiological issues such as water temperature and/or quality, general physiology of an animal, animal size, and numerous other factors that cannot be quantified or predicted. For example, fish of the same species but of different sizes could be affected by intense sounds differently.

D. Pathology and histopathology

Results indicate that LFA exposure at the levels described in this study caused no acute gross- or histopathology in rainbow trout. There were no changes in hematocrit between control and any of the LFA-exposed animals. LFA exposure did not cause shearing of red blood cell membranes as indicated by a lack of sanguineous color in the plasma. The rainbow trout is called a physostome because it has an esophageal connection to the swim bladder (i.e., a pneumatic duct) and is thus able to exchange gas from within the swim bladder directly through the mouth. It would be interesting to observe possible changes in a species that does not have a connection between the esophagus and the swim bladder (i.e., physoclistus condition without a pneumatic duct). Without the ability to "burp" swim bladder gas upon traumatic sound wave impingement, it may be more likely to observe changes in the microvasculature of the swim bladder and other organ systems.

The histological findings are in contrast to other studies using explosives or pile driving, many of which are in the gray literature, that suggest damage to the swim bladder and other body tissues because of exposure to explosives (e.g., Yelverton *et al.*, 1975; Sverdrup *et al.*, 1994; reviewed in Hastings and Popper, 2005). It is very hard, however, to compare the LFA results with results from other types of acoustic stimuli since the sounds are so notably different.

E. Inner ear morphology

The sensory epithelia and sensory hair cells showed no apparent morphological damage after any of the LFA exposures, even though there were shifts in hearing thresholds. However, it should be noted that tip link integrity was not accessed for this project. Results of the ultrastructural analysis using scanning electron microscopy suggest that the LFA sonar sounds do not cause gross morphological damage to the sensory epithelia or sensory hair cells in the inner ear. The effect LFA sounds might have on the physiological or metabolic changes of the sensory hair cells themselves is unknown and would be difficult to determine.

F. Effects of SURTASS LFA on fish

A single LFA sonar signal used in this study was made up of three parts. The LFA signal is turned on for 108 s, followed by a quiet of period of 9 min. This "signal followed by quiet" is presented three times, giving a total of 324 s of signal exposure for our MAX tests and 648 s of signal exposure for our MAX*2 tests.

Typical use of LFA sonar is on a ship moving about 3 knots (1.5 m/s). The critical issue from these studies is whether LFA sonar exposure impairs the survival of fishes and, more important, whether survival would be impaired in a normal environment when a ship using LFA is in the vicinity of a fish. In answering this question, several factors must be taken into consideration.

First, the sound level to which fish were exposed in these experiments was 193 dB re 1 μPa^2, a level that is only found within about 100 m of a ship using LFA. Thus, the likelihood of exposure to this or higher sound levels is small, considering all the possible places a fish might be relative to the sound source. In effect, the area of the ocean ensonified by a single LFA source at 193 dB re 1 μPa^2 or higher is small compared to the ocean area ensonified by the LFA source at lower sound levels.

Second, the presentation of the LFA sound in this study represents substantially longer exposure than might be encountered by fishes in the wild because during actual LFA use, the source is on a moving ship. An absolute worst-case scenario would be a fish following the ship during ensonification however, this is very unlikely. A realistic worst-case scenario is a stationary fish. Assuming that the ship is moving at 3 knots (the general speed of ships with the LFA device) and ensonifies a stationary fish at 193 dB, the fish would be exposed to the maximum sound level for only a few seconds. The next received exposure of the stationary fish would be at a significantly lower sound level since the ship would have moved on during ensonification and during the quiet period for the sonar.

The results presented here are representative of a worst-case scenario (albeit highly unlikely) of hearing for fish following exposure to LFA sonar. These results represent two extremes of effects—a minimal effect of 5-dB threshold shifts to a large effect of 20-dB threshold shifts at a single frequency that may or may not recover. The temporary exposure to sounds of SURTASS LFA sonar does affect rainbow trout auditory thresholds, and how this deficiency in hearing at one frequency impacts their ability to survive in the wild is unknown. Ultimately, the results showed no mortality associated with sound exposure and all fish appeared healthy and active until the end of our experimental week, when they were sacrificed or returned to the fish farm from which they were purchased.

The rainbow trout exhibited a behavioral response to the onset of the LFA signal, the description of which is presented elsewhere, but included a rapid burst of swimming immediately after the sound onset (Wysocki, private communica-

tion). How such responses may affect a fish's long-term behavior and survival and the potential cumulative effects of repeated exposure are not known.

G. Relevance to other anthropogenic sources

The relevance of these studies to other high-intensity sound sources is tenuous. In particular, the LFA sound is ramped on relatively slowly and consists of FM sweeps and pure tones. In contrast, other anthropogenic sources such as pile driving and seismic air-guns have rapid onsets and may have significantly different effects on fish due to their transient nature. Thus, extrapolation from LFA to other sources should be done with the utmost caution.

At the same time, it may be possible to compare exposures using SEL as a measure of total energy in 1 s of exposure (see Hamernik and Hsueh, 1991; Finneran *et al.*, 2002; Hastings and Popper, 2005). The SEL provides a measure of the total energy in 1 s of a signal, and this is a more appropriate and encompassing comparison between signals of different types (e.g., air-gun versus sonar) than is peak or rms pressure, particularly for very short signals with significant energy peaks. SEL is calculated by summing the cumulative pressure squared (p^2) over time and is expressed as dB re 1 μPa^2 s.

In this study, the 1-s SEL was 189 dB re 1 μPa^2 s. To date, the only other report of SEL for fish was in exposure to a seismic air-gun (Popper *et al.*, 2005), where the maximum SEL to which another salmonid, the broad whitefish (*Coregonus nasus*), was exposed was about 180 dB re 1 μPa^2 s. The maximum peak pressure in that study was around 197 dB re 1 μPa^2, a level considerably above that used in this study, but that signal was of a lower SEL calculation. In that seismic study, exposure to relatively high SEL level signals resulted in little or no physiological effects on fish, suggesting that salmonids are potentially able to withstand higher sound levels than have been tested to date.

It is also hard to compare the exposure to the LFA signal to the effects of generally elevated background noise because of increased shipping and other sources. It is known that increased background noise can have long-term effects on hearing sensitivity in fishes that have auditory specializations (Smith *et al.*, 2004a, b), but unless the sound was exceedingly loud, it may not affect hearing in fishes, such as salmonids, that do not have such specializations (Smith *et al.*, 2004a). However, noise can also affect other aspects of fish physiology, e.g., ship noise has been shown to induce increased cortisol secretion (i.e., increases stress levels) in different species of freshwater fishes regardless of their hearing sensitivity (Wysocki *et al.*, 2006).

H. Extrapolation to other species

As mentioned earlier, the effects of LFA sonar on salmonids are of concern, but most are listed or endangered species. Thus, this study used rainbow trout as a representative salmonid. Among most salmonid species, the ears, lack of auditory specializations, and hearing sensitivity are all very similar to those of rainbow trout.

Extrapolation of these rainbow trout results to other nonsalmonids or fish with hearing specializations is not possible since there are differences in ear structures and hearing sensitivity between various groups. Studies need to be performed on several other species (these are planned for the near future) to even consider extrapolation to other species or groups of fish.

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