Group behavior and tolerance of Eurasian minnow (*Phoxinus phoxinus*) in response to tones of differing pulse repetition rate[a]

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ABSTRACT:

Behavioral guidance systems are commonly used in freshwater fish conservation. The biological relevance of sound to fish and recorded responses to human-generated noise supports the viability of the use of acoustics as an effective stimulus in such technologies. Relatively little information exists on the long-term responses and recovery of fish to repeated acoustic exposures. In a controlled laboratory study, the response and tolerance of Eurasian minnow (*Phoxinus phoxinus*) shoals to tonal signals (150 Hz of 1 s pulse duration) differing only in temporal characteristics ("continuous," "slow," "intermediate," or "fast" pulse repetition rate) were investigated. In comparison to independent control groups, fish increased their mean group swimming speed, decreased inter-individual distance, and became more aligned in response to the onset of all four acoustic treatments. The magnitude of response, and time taken to develop a tolerance to a treatment differed according to pulse repetition rate. Groups were found to have the greatest and longest lasting response to tone sequences tested in this study when they were pulsed at an intermediate rate of $0.2 \, \text{s}^{-1}$. This study illustrates the importance of understanding the response of fish to acoustic signals, and will assist toward the development of longer-term effective acoustic guidance systems.

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I. INTRODUCTION

Aquatic animals gain vital information from the acoustic signals present within their environment (Hawkins and Myrberg, 1983). Natural soundscapes facilitate a variety of survival functions in fish, including navigation [e.g., Apogonidae *sp.* reef settlement, Simpson *et al.* (2005)], selection of mates [e.g., Pomacentridae *sp.* courtship calls, Mann and Lobel (1997)], conspecific interactions [e.g., *Amphichthys cryptocentrus* male competition, Salas *et al.* (2018)], and prey seeking and predator avoidance (Hawkins and Popper, 2018; Ward *et al.*, 2011). The responses of fish to anthropogenic noise (e.g., shipping, naval sonar transmissions, pile driving) are also widely described. Man-made sources of noise mask communicative signals (de Jong *et al.*, 2018), and alter coordinated movement (Herbert-Read *et al.*, 2017), spatial distribution (Currie *et al.*, 2020), and orientation and cohesion of groups (Herbert-Read *et al.*, 2017). Evidently, the ubiquitous nature of anthropogenic noise in the environment is cause for concern, with a range of responses having been observed in fish.

The biological relevance of sound to fish and observed responses to human-generated noise support the use of acoustics as a viable mitigation tool in freshwater fish conservation (Popper and Carlson, 1998). Acoustic guidance systems are deployed to reduce fish impingement on screens designed to prevent ingress into water intakes (Maes *et al.*, 2004) and divert individuals to safer routes of passage [e.g., *Alosa pseudoharengus*: Dunning *et al.* (1992); *Salmo salar*: Scruton *et al.* (2003); *Anguilla anguilla*: Deleau *et al.* (2019), Piper *et al.* (2019)], and control range expansion of invasive species [e.g., *Hypophthalmichthys molitrix*, Vetter *et al.* (2015)]. For effective screening, targeted fish must be able to detect and localise a sound source above background noise, and subsequently elicit a desirable response. Importantly, the acoustic stimuli also must remain effective with repeated exposure over time (Blumstein, 2016). While interest in the use of sound to control the movement of fish has been investigated since the late 1940s (Burner and Moore, 1962), relatively little information exists on how the behavior of freshwater fish changes in response to repeated exposure to acoustic signals over time.

Understanding behavioral mechanisms that underpin fish responses to human-generated acoustic stimuli is important in informing sustainable management strategies (Blumstein, 2016). A novel acoustic stimulus may initially provoke a substantial anti-predator response (Voellmy *et al.*, 2016), but after multiple presentations an instantaneously demonstrable behavioral state (measured at a point in time) known as tolerance may occur, resulting in a short-term

decline in the frequency or magnitude of the response (Bejder *et al.*, 2009). Without adequate recovery time, or with a series of frequent exposures over time, the degree of tolerance may change and the longer-term process of habituation could occur, after which responses to the same stimuli cease to be observed (Bejder *et al.*, 2009; Blumstein, 2016). Rate of recovery may, however, be influenced by the temporal characteristics of the signal (e.g., pulse repetition rate, pulse repetition interval, amplitude ramp-up). Studies have investigated these effects on marine fish species (Neo *et al.*, 2014; Neo *et al.*, 2015b) as anthropogenic broadband noise sources are highly variable in their temporal structure. For example, European seabass (*Dicentrarchus labrax*) were noted to recover more quickly after exposure to continuous than impulsive noise stimuli (Neo *et al.*, 2014). Nevertheless, better understanding of tolerance and habituation-like processes (Geffroy *et al.*, 2015), or recovery of freshwater fish in response to acoustic signals that differ in temporal characteristics, warrants further investigation. This will aid in the development of more effective behavioral guidance systems and further assist conservation efforts to reduce the negative impacts of anthropogenic noise.

The aim of this laboratory study was to investigate the influence of pulse repetition rate on group behavior and tolerance of a freshwater shoaling fish. A total of 250 fish were tested across 50 independent trials. Groups of five individuals were independently exposed to one of four acoustic treatments ("continuous," "slow," "intermediate," or "fast" pulse repetition rate) or a "silent" ambient control. The investigation focused on three group behaviors: (1) swimming speed (ms^{-1}); (2) inter-individual distance (m); and (3) alignment (°). Building on work conducted on marine species [e.g., Neo *et al.* (2014), Neo *et al.* (2015b), and Neo *et al.* (2018)], it was hypothesized that time taken to develop a tolerance to an acoustic stimulus, or return to baseline behavior, would be greater for groups exposed to tones with longer intervals between pulses. The Eurasian minnow (*Phoxinus phoxinus*), was selected as a model species because of its facultative shoaling behavior, and local abundance.

II. METHODS

A. Fish collection and husbandry

In April 2017, 270 adult Eurasian minnows were collected with a seine net from the River Itchen navigation channel, Hampshire, UK (51°02′58.9″N 1°18′42.2″W). The freshwater habitat from which fish were sourced averaged 15 m in width and depths ranged from 0.34 to 0.94 m. The watercourse is highly modified, with the combination of shallow-water (often <1 m depth) and man-made solid banks, contributing to a complex acoustic environment.

Fish were transported to the University of Southampton's ICER facilities and gradually introduced to a holding net (0.78 m × 0.3 m × 0.62 m; water depth: 0.45 m; stocking density: 5.98 kg/ m^{-3}) within a tank (1.5 m × 1.0 m × 0.78 m; water depth: 0.68 m; mean temperature ± SE: 13.7 ± 0.3 °C) over a period of three hours. Minnow were acclimatized for five days prior to the start of the experiments, and water quality was monitored and maintained (NO^{3-}: < 50 mg L^{-1}; NO^{2-}: < 1 mg L^{-1}; NH$_3$: 0; and pH: < 8.4). Fish were kept on a 14:10 h light:dark photoperiod cycle and fed daily with commercially available aquarium food until satiation. After each trial, fish were measured (standard length ± MAD: 57.2 ± 4.4 mm) and weighed (wet mass ± MAD: 2.8 ± 0.9 g). Differences (Kruskal-Wallis rank sum) in wet mass (χ^2 = 14.69; *d.f.* = 4; *p* < 0.01) and standard length (χ^2 = 11.54; *d.f.* = 4; *p* < 0.05) were apparent between treatments. However, *post hoc* Dunn's test indicated deviations between treatments to be only for larger fish exposed to the "SLOW" treatment (Table I). Experiments were performed after review and approval by the University of Southampton's Animal Welfare and Ethical Review Board.

B. Experimental setup

Experiments were conducted within an acoustically isolated room, where trials were conducted in a physically segregated section (86 cm × 30.8 cm × 30.2 cm) of a still-water acrylic tank (300 cm × 30.8 cm × 30.2 cm; wall thickness: 1.2 cm) (Fig. 1). Use of a still water tank-based approach allowed control over experimental conditions to ensure sound was the sole external stimuli of influence. Acoustic testing in a small tank is more appropriate for riverine freshwater species than marine fish, since the acoustic environment in a tank is closer to that of a shallow river than of typical marine environments (Tonolla *et al.*, 2010). That is not to suggest that even for these freshwater species that results from tank tests can be transferred directly to naturally occurring environments without further test or validation.

Two fully immersed speakers (Electro-Voice UW-30; maximal output 153 dB re 1 μPa at 1 m for 150 Hz, frequency

TABLE I. Treatment parameters and acoustic conditions encountered by *Phoxinus phoxinus* in experimental trials conducted to assess their behavioral response and tolerance to tones differing in pulse repetition rate.

Treatment	n trials	Standardized tone SPL (RMS) (dB re 1 μPa)	Pulse repetition interval (s)	Pulse repetition rate (s^{-1})	Pulse duration (s)	Median fish standard length ± MAD (mm)	Median fish wet mass ± MAD (g)
CONTROL	10	na	na	na	na	58.0 ± 8.9	2.8 ± 1.3
CONTINUOUS	10	155	na	na	na	58.0 ± 5.2	2.8 ± 0.7
FAST	10	155	2.0	0.5	1.0	55.0 ± 3.0	2.4 ± 0.6
INTERMEDIATE	10	155	5.0	0.2	1.0	57.0 ± 3.0	2.8 ± 0.6
SLOW	10	155	10.0	0.1	1.0	59.5 ± 8.2	3.3 ± 1.5

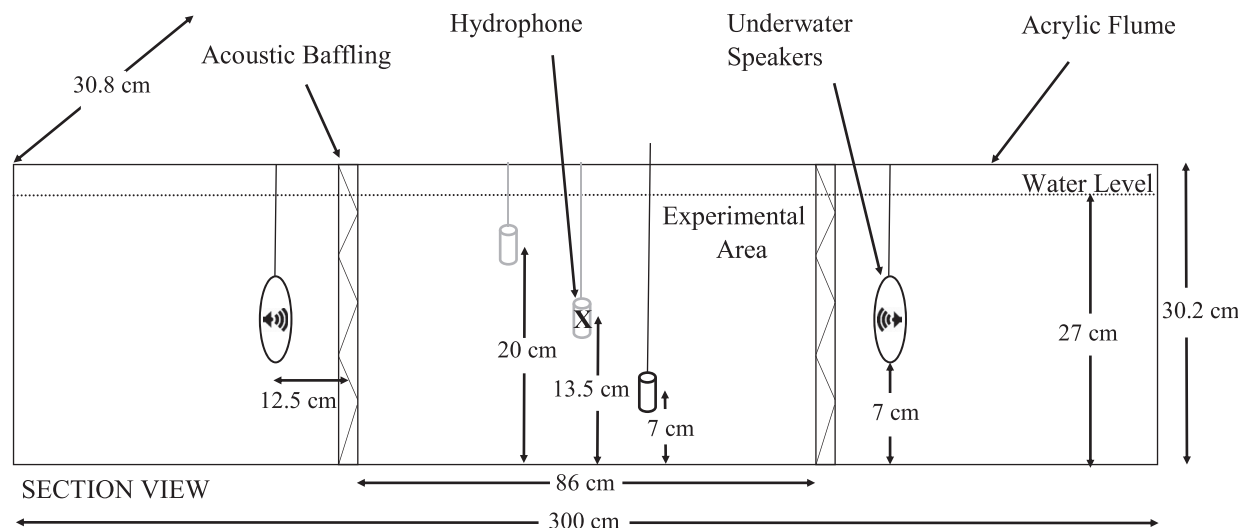1710   J. Acoust. Soc. Am. **147** (3), March 2020

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FIG. 1. Schematic of experimental flume setup including the experimental area in which fish were exposed to acoustic stimuli. Hydrophone positions are shown for acoustic mapping at three water depths (7 cm; 13.5 cm; 20 cm), with "X" indicating the position at which sound pressure levels (RMS) (dB re 1 μPa) were standardized.

response 0.1–10 kHz; Lubell Labs, Columbus, OH) were used to generate the sound field. Speakers were positioned at a fixed point in the middle of the water column, with one behind each of two micro-mesh acoustic baffles at either end of the experimental area. Water was maintained at a constant depth of 27 cm and replaced every ten trials to remove build-up of biological debris, or residual chemical alarm substance ("Schreckstoff," Hasan, 2018). Tank water was left to settle overnight, allowing for a return to room temperature (mean ± SE: 14.0 ± 0.17 °C) and the release of gas bubbles which may influence the acoustic environment.

Fish within the experimental area were visually isolated from the experimenter by plastic blackout sheeting. Light levels were maintained using a white background, attached to the outside of the experimental area and lit from underneath by two PhotoSEL Photography bulbs (pure white full-spectrum flicker free; 85 W, 5000 lumen; SJT Commercial Ltd., UK). Consistent lighting throughout the trials allowed for sufficient contrast of the fish for digital video recordings using a webcam (C920; HD 10809; 30 frames per second; Logitech Pro, Switzerland) mounted overhead.[1]

C. Sound stimuli and acoustic mapping

A sinewave frequency of 150 Hz has previously been observed to elicit changes in Eurasian minnow group behavior (e.g., startle response, group swimming speed, shoal distribution) (Currie *et al.*, 2020), and as a result was subsequently chosen for this experiment. Sound samples were produced through the use of custom written MATLAB script (Release 2017a, The Mathworks, Inc., Natick, Massachusetts, United States). The signal was produced from a laptop computer via a DAQ (NI USB-6341; National Instruments, UK) and played from the underwater speakers through a ROTEL RA-920AX amplifier (75 W, frequency response range approximately 0.02–20 kHz; Rotel Europe, UK). Four acoustic treatments were used in the experiments to cover a range

of inter-pulse (5 ms ramp-up/ down Hanning taper) spontaneous recovery times (Fig. 2; Table I). A "control" of no sound (ambient noise: 72 dB re 1 μPa) was also tested, where an electrical signal was sent to the speakers to avoid any potential confounding influences (e.g., electroreception). The sound pressure level (SPL) [root-mean-square (RMS)], calculated for the dominant frequency of treatment stimuli was standardized in the centre of the experimental area (155 dB re 1 μPa). Note that although the RMS SPL was constant over any time window for which the sound was on (barring start-up transients in the first 5 ms), the total amount of acoustic energy delivered during a trial decreases as the off-time increases during a trial of fixed duration (as opposed to a trial that delivers a fixed number of pulses). A 1 s pulse duration was used for all pulsed exposures, and since sound travels approximately 1.5 km in this time, the pulse duration is sufficiently long to build up the same reverberant sound field in each case (Fig. 2).

In advance of conducting trials, the acoustic environment was quantified for the tonal stimuli (Fig. 3). Acoustic measurements were made at 306 positions (17 × 6 × 3 grid) using a hydrophone (type: 4013: manufacturer-calibrated sensitivity −211 dB re: 1 V μPa^{-1}, frequency response 0.001–170 kHz; Teledyne RESON, Slangerup, Denmark) fixed to a customized rig, and connected to a hydrophone voltage amplifier (type: A1001; 9 V; gain +40 dB, high pass filter 100 Hz; etec, Frederiksværk, Denmark). The signal was recorded on a laptop via the DAQ, controlled using a custom written MATLAB script (sampling rate 25.6 kHz). A pistonphone (type: 4229; Brüel & Kjær, UK) was used to ensure hydrophone calibration. Resulting SPLs were used to describe the sound-field within the tank across three different depths [Fig. 3(B)]. The particle acceleration component, *a*, was calculated based on

$$a = -\frac{1}{\rho}\nabla P, \qquad (1)$$
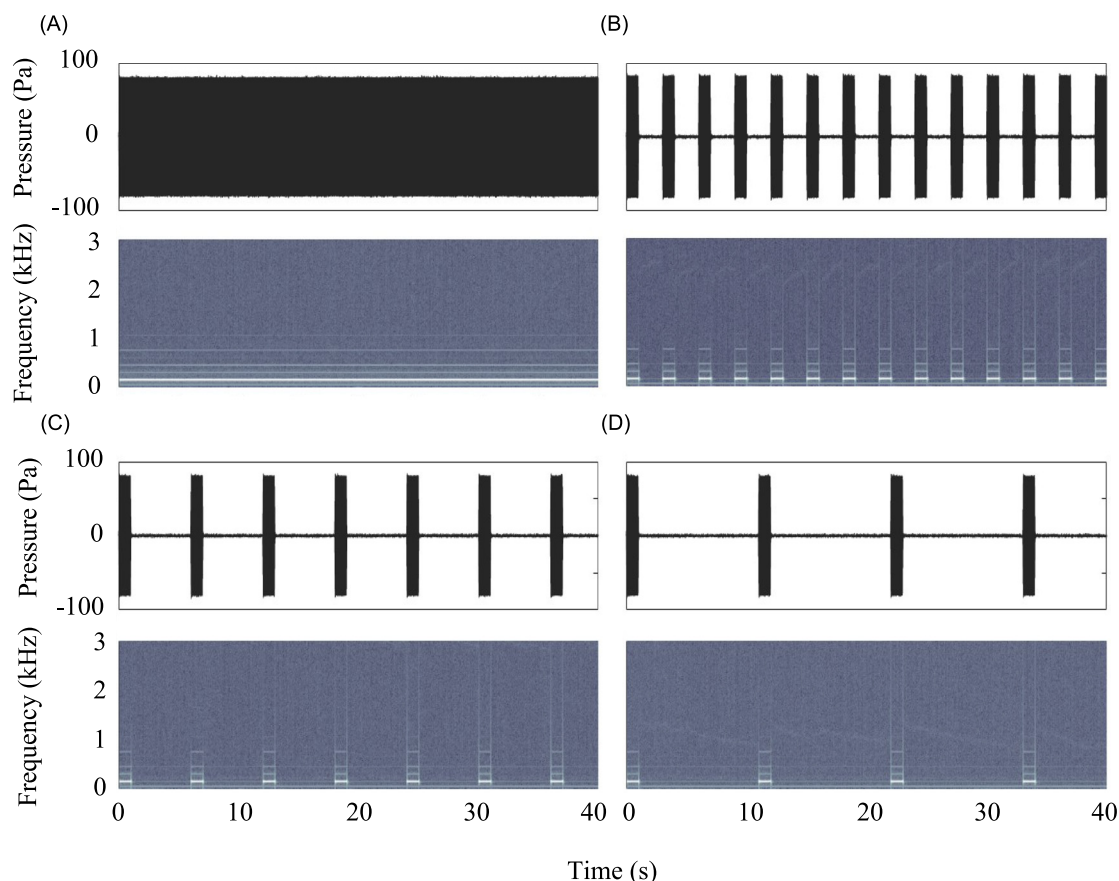
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FIG. 2. (Color online) Waveforms and spectrograms (dB re 1 $\mu Pa^2\,Hz^{-1}$) showing temporal structure of acoustic treatment stimuli: (A) CONTINUOUS; (B) FAST (PRR: $0.5\,s^{-1}$); (C) INTERMEDIATE (PRR: $0.2\,s^{-1}$); and (D) SLOW (PRR: $0.1\,s^{-1}$) (sampling rate: 25.6 kHz; FFT 2048; overlap 50%; Hamming Window; frequency range 0–3 kHz).

where P is the complex pressure amplitude and ρ is the ambient density.

As the pressure amplitude and phase were measured on a regular grid of points, from these measurements, the complex pressure amplitude at each was computed. The pressure gradient was approximated using finite differences in all three directions (x, y, and z). The particle acceleration could then be determined by taking the real part of Eq. (1). The amplitude of the acceleration was computed as the square root of the sum of the three directional components squared. Particle acceleration (dB re $1\,mm\,s^{-2}$) for the centre depth (13.5 cm) of the tank was then mapped [Figs. 3(C) and 3(D)].

The nature of the near-field conditions within a small tank relative to wavelength were responsible for highly complex and directionally variable conditions (Gray *et al.*, 2016). High levels of particle motion exist within small-tank setups, and in this experiment, a reduction in particle acceleration was recorded with increasing distance from the two speakers. It should be noted, however, that while acoustic stimuli were standardized in the centre of the experimental area, the left-hand speaker had a moderately stronger influence on the sound field. The acoustic field within the central region of the experimental area was composed of higher SPLs and lower particle acceleration, an effect of interference from the directionally opposing speakers, or perhaps the result of a standing wave.

D. Experimental protocol

A total of 50 trials were conducted, ten replicates for each treatment and control. Each trial lasted a total of 60 min, including a 20 min acclimation period. After this time, an acoustic playback treatment was turned on for 20 min, projected simultaneously from two underwater speakers. Control groups of fish were independently tested under the same conditions, but without the acoustic playback stimuli. A random number generator was used to determine order of playback, thereby avoiding order effects. A final post-treatment period of 20 min was recorded after each treatment.

Each trial consisted of five naive fish (total n = 250), introduced simultaneously as a group to the centre of the experimental area. This avoided confounding influences on the order of introduction and left-right, or other spatial sampling bias. The 60 min video recording was then started.[1] Each group of five fish was used once only.

E. Behavioral parameters and statistical analysis

Fish were tracked using a custom written MATLAB script. The mean shoal centre [$Xc(n)$] location (x, y axis) of groups of fish were taken for each frame. $Xc(n)$ was calculated as the position of the ith fish in the nth video frame [vector $\underline{X}_i(n) = (x_i(n), y_i(n))^t$], where $x_i(n)$ corresponds to the
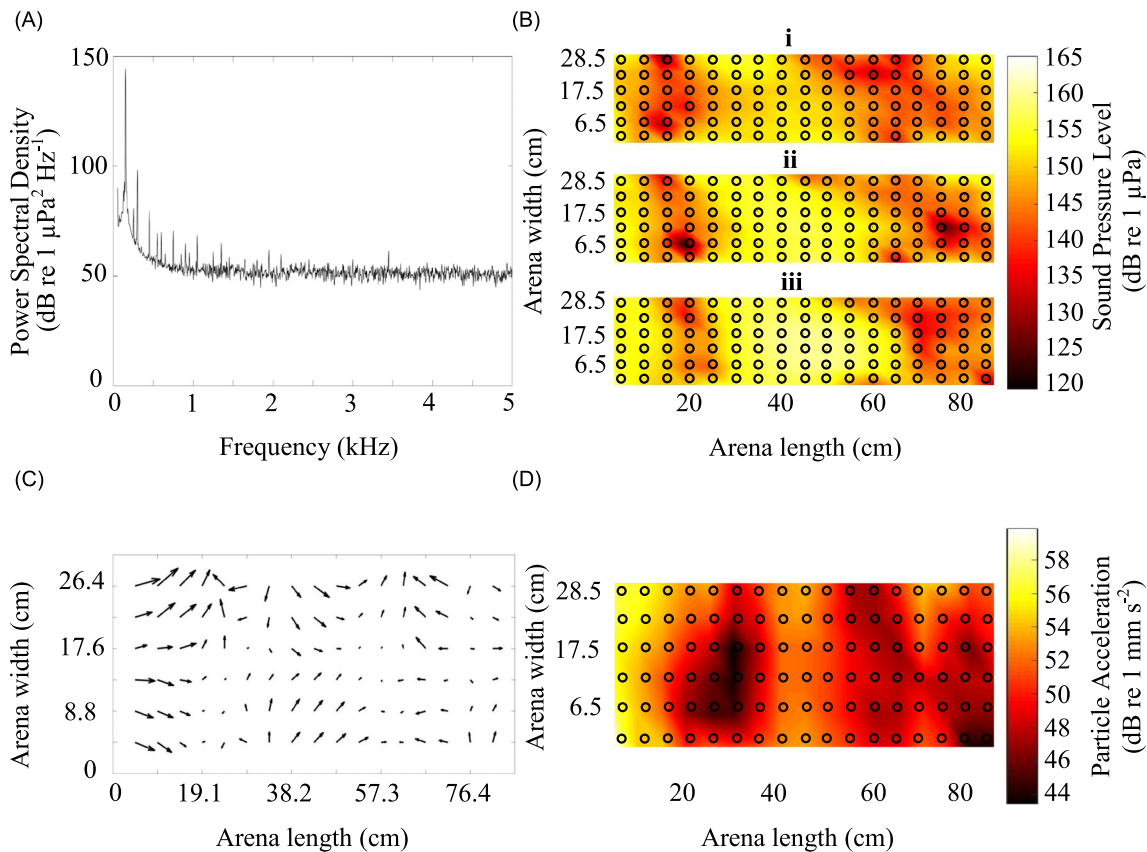
FIG. 3. (Color online) (A) Example power spectral density (dB re 1 μPa^2 Hz^{-1}) of acoustic conditions within the experimental area (sampling rate 25.6 kHz; FFT size 8192 (3 Hz bin width), overlap 91.5%, von Hann Window, frequency range 50–5000 kHz); (B) heat maps of sound pressure levels (SPL) (dB re 1 μPa) (150 Hz sinewave) for acoustic stimuli across three depths (i) 7 cm; (ii) 13.5 cm; and (iii) 20 cm; (C) quiver map indicating particle acceleration (dB re 1 mm s^{-2}) directionality at 13.5 cm depth; and (D) heat map of particle acceleration (dB re 1 mm s^{-2}) at 13.5 cm depth. Note: open circles indicate the location of the hydrophone when measuring the sound field.

distance along the length of the tank, and $y_i(n)$ to the breadth, so as $\underline{X}_C(n) = (x_c(n), y_c(n))^t = (\underline{X}_1(n) + \underline{X}_2(n) + \underline{X}_3(n) + \underline{X}_4(n) + \underline{X}_5(n))/5]$. Group behavior was then calculated as (1) group mean swimming speed (ms^{-1}), computed from $Xc(n)$ over time; (2) inter-individual distance (m), defined as the mean distance from $Xc(n)$, with measurements taken from the central point of each fish; and (3) alignment (°), as the standard deviation of the angle of the fish compared to one another. This provided an output of 108 000 data points per variable calculated for each trial (n = 50).

As data failed to meet the assumptions of normality (Shapiro-Wilks test) and homoscedasticity (Levene's test), it was log$_{10}$ transformed to meet prerequisites for use in parametric analysis. Statistical analysis was performed with freeware programme RSTUDIO v3.2.2 (The R Foundation for Statistical Computing, 2015).

To assess whether group behavior changed during the trials, repeated measures analyses of variance (ANOVAs) were performed. Treatment was allocated as a between-subjects factor, and time period as a within-subjects factor for group swimming speed, inter-individual distance, and alignment. Time was divided into seven blocks of five-minute bins. Differences in behavior over time were investigated between "pre-" (five minutes immediately pre-stimuli), "during-" (first;

middle; and final five minutes during playback: Figs. 4 and 5), and "post-exposure" phases (first; middle; and final five minutes post-stimuli). Addition of a baseline control within the analyses increased the probability that any main effects would be outperformed by interaction effects. Subsequently, when these occurred, each treatment was separately tested for differences over time using repeated measures ANOVAs.

The univariate approach of repeated measures ANOVAs (sphericity) was assessed using Mauchly's test (Huynh and Mandeville, 1979). Greenhouse-Geisser corrections (Greenhouse and Geisser, 1959) were utilized for epsilon (ε) values less than 0.75; and Huynh-Feldt corrections (Huynh and Feldt, 1976) for values greater than 0.75, when sphericity could not be assumed. When between factor effects were apparent, they were investigated using *post hoc* Bonferroni tests.

Treatment effects of each behavioral parameter were plotted as a 10 s running t-statistic over time. An independent two-sample t-test with assumed equal variance was used to compare each treatment group (n = 10) to control "baseline" fish (n = 10). An alpha-level of 0.05 was used to indicate a significant influence of treatment. Note that owing to natural variation in fish behavior, deviations may occur between treatment and control fish prior to the onset of an
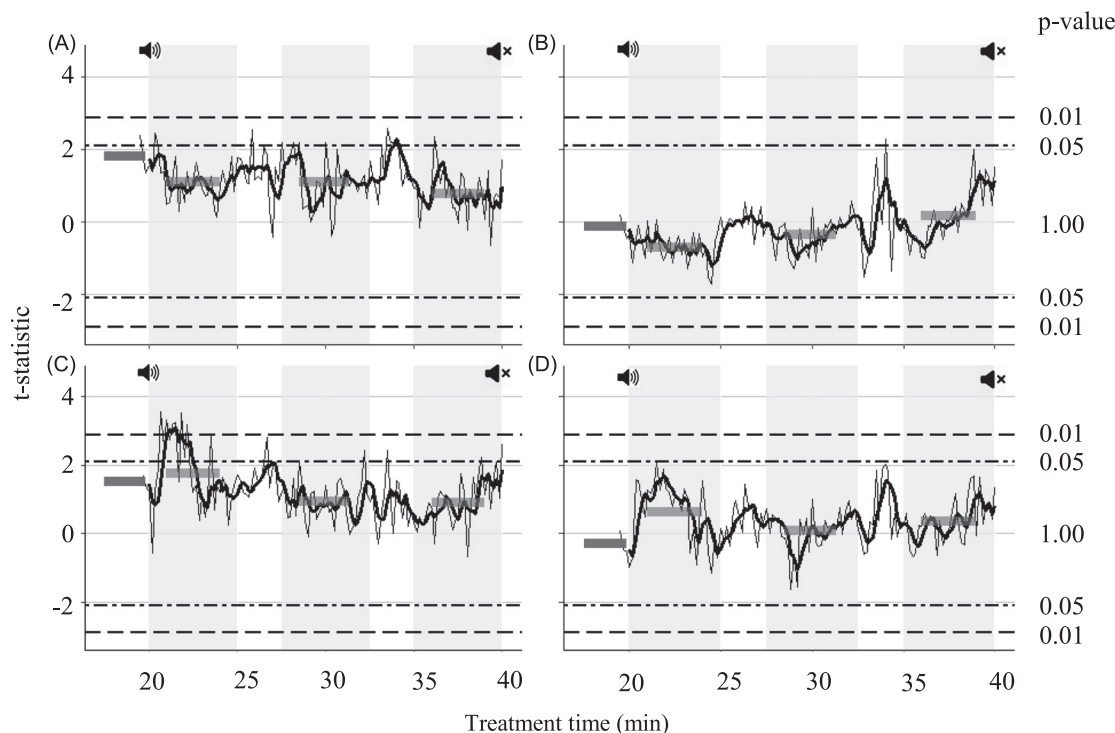
FIG. 4. Change in speed (ms^{-1}) of Eurasian minnow exposed to (A) CONTINUOUS; (B) FAST; (C) INTERMEDIATE; and (D) SLOW pulse repetition rates, compared to baseline unexposed control fish groups. **Note**: Continuous thin solid lines represent 10 s running t-statistics; and the thicker solid line, a four-point central moving average of the 10 s t-statistic (for assessment of tolerance). Horizontal dot-dashed lines indicate a cut-off at $p = 0.05$, and dashed lines a $p = 0.01$ for $\alpha = 0.05$ (in both directions). The four thick horizontal bars represent averages (one pre-exposure, and three during-exposure) over time of 5-min bins (areas in grey represent blocks for repeated measures ANOVA assessing change in speed over time). The speaker with "waves" portrays sound on, and the speaker with an X, sound off.

acoustic exposure. Using a four-point central moving average of the 10 s running t-statistic, where an influence was observed within the first five minutes of acoustic exposure, the total duration from surpassing the significance threshold ($p < 0.05$) to pre-threshold recovery level ($p > 0.05$) of this influence, or "time to tolerate" was calculated.

III. RESULTS

A. Swimming speed

Swimming speed declined at the onset of acoustic stimuli for all treatments, reflective of a freezing behavior, where most minnow groups exhibited temporary cessation of body movement (Fig. 4). The three impulsive acoustic treatments (SLOW, INTERMEDIATE, and FAST) induced a higher swimming speed over time in comparison to acclimated pre-exposure levels (Wilks' Lambda = 0.4; $F_{3.61, 162.65} = 12.1$; $p < 0.01$; $\eta^2 = 0.21$). Groups exposed to CONTINUOUS tones, however, maintained a relatively consistent speed after the initial decline. Although changes in speed significantly differed from control groups of fish, the effects observed between treatments did not. An interaction between treatment and time did exist (Wilks' Lambda = 0.39; $F_{11.32, 162.65} = 2.2$; $p < 0.01$; $\eta^2 = 0.16$). Furthermore, the elevated swimming speed was observed to continue into the post-exposure phase, after termination of acoustic stimuli.

The INTERMEDIATE acoustic pulses were observed to induce the longest significant deviation from control group baseline speed, with time taken to develop a tolerance at the beginning of the sound exposure period taking 2 min 18 s [$p < 0.01$; Fig. 4(C)]. The same effect was observed in response to SLOW pulses, however, the greater shift in speed did not pass the arbitrary threshold for significance. Time taken to develop a tolerance to acoustic stimuli also could not be quantified for CONTINUOUS and FAST treatments.

B. Inter-individual distance

In response to the onset of all acoustic treatments, fish shoals initially increased cohesion, displayed by a decrease in inter-individual distance, however, this did not differ to the control fish (Fig. 5). The distance between individuals gradually increased over time, with minnow spreading further apart from one another (Wilks' Lambda = 0.49; $F_{3.80, 171.15} = 8.2$; $p < 0.001$; $\eta^2 = 0.15$).

Minnows took longer to develop a tolerance to the INTERMEDIATE treatment, returning to baseline inter-individual distance after 2 min 18 s [$p < 0.01$; Fig. 5(C)] of acoustic exposure. Inter-individual distance was also greater than control groups for minnow exposed to the SLOW treatment, with the change in behavior returning to below significance thresholds after 59 s [$p < 0.05$; Fig. 5(D)]. The influence of FAST and CONTINUOUS pulse repetitions on
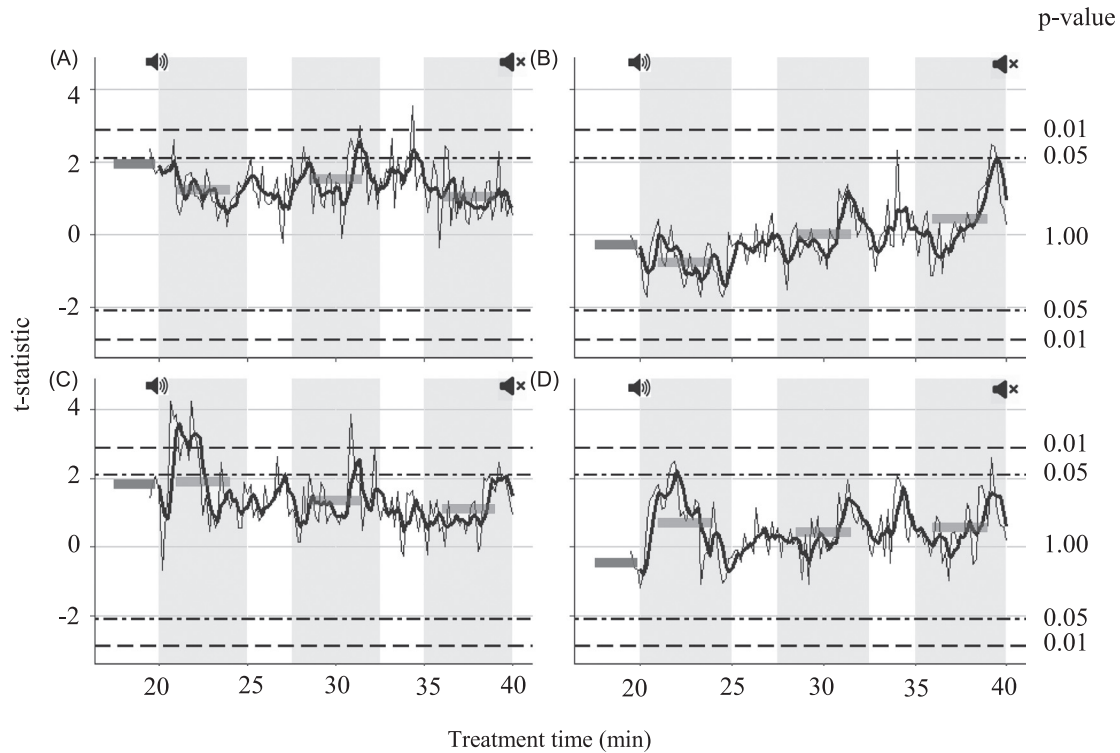
FIG. 5. Change in inter-individual distance (m) of Eurasian minnow exposed to (A) CONTINUOUS; (B) FAST; (C) INTERMEDIATE; and (D) SLOW pulse repetition rates, compared to baseline unexposed control fish groups. **Note**: Continuous thin solid lines represent 10 s running t-statistics; and the thicker solid line, a four-point central moving average of the 10 s t-statistic (for assessment of tolerance). Horizontal dot-dashed lines indicate a cut-off at p = 0.05, and dashed lines a p = 0.01 for $\alpha = 0.05$ (in both directions). The four thick horizontal bars represent averages (one pre-exposure, and three during-exposure) over time of 5-min bins (areas in grey represent blocks for repeated measures ANOVA assessing change in inter-individual distance over time). The speaker with "waves" portrays sound on, and the speaker with an X, sound off.

inter-individual distances were not large enough to interpret a "time to tolerate."

C. Alignment

Although alignment did not change significantly over time, groups were observed to exhibit an increase in alignment among individuals at the onset of the stimuli in comparison to control groups. Over the exposure period, individuals gradually became less commonly aligned, but again this effect was not significant. The observed effect was significant between treatments ($F_{4,45} = 4.05$; $p < 0.01$; $\eta^2 = 0.26$), with *post hoc* analysis indicating that fish became less well aligned under FAST repetitions compared with those under the SLOW repetition treatment ($p < 0.01$). For this metric, however, the time taken to tolerate could not be quantified, since no significant changes from the control group were observed.

IV. DISCUSSION

This study investigated the group behavior and tolerance of Eurasian minnow to tonal acoustic stimuli that differed with respect to pulse repetition rate (PRR). At the onset of all acoustic treatments, higher shoal swimming speed, lower inter-individual distance, and greater alignment, was observed compared to control groups. Eurasian minnow have previously been observed to exhibit similar

group behavior in response to a low frequency (150 Hz) tonal stimuli (Currie *et al.*, 2020), and the observed behavioral changes are consistent with several other studies conducted on fish using varying broadband noise sources (e.g., Herbert-Read *et al.*, 2017; Neo *et al.*, 2014). Notably, our study additionally observed that PRR influenced the time taken to tolerate a tonal stimulus in freshwater fish shoals.

Changes of swimming speed among individuals within a group may be used by conspecifics to gather information on their surrounding environment (Harpaz *et al.*, 2017). Increases in swimming speed may indicate fright or anxiety associated with a perceived threat (e.g., predation risk: Neo *et al.*, 2014), and changes are highly correlated with alterations in group inter-individual distance and alignment, or orientation (Kent *et al.*, 2019). An increase in inter-individual distance, as observed over time during this study, may be costly, with isolated individuals more susceptible to predation (Handegard *et al.*, 2012). Noise can distract from the detection of an additional stimulus, and therefore reduce information sharing. For example, fathead minnow (*Pimephales promelas*) detection of a conspecific chemical alarm cue is reduced in the presence of anthropogenic noise (Hasan *et al.*, 2018). As for inter-individual distance, group alignment enables individuals to gain information from others (Harpaz *et al.*, 2017). For example, increased alignment among shoal members is linked to the detection of a predatory threat (Herbert-Read *et al.*, 2017). This behavior

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was observed at the onset of all acoustic stimuli, but group alignment rapidly decreased over time.

Impulsive anthropogenic noise induces greater initial and delayed behavioral changes in fish than continuous sound differing only in its temporal characteristics (PRR) (Neo *et al.*, 2014; 2015b; Sabet *et al.*, 2015). In this study, INTERMEDIATE repetitions were observed to elicit the greatest and longest lasting difference in response across all tested parameters in comparison to control fish. Minnow were least tolerant of this treatment, which induced a greater group swimming speed and inter-individual distance, and lower alignment. This result is similar to those observed in marine studies that exposed European seabass [*Dicentrarchus labrax*: Neo *et al.* (2014), Neo *et al.* (2015b), Neo *et al.* (2018)] to broadband noise, in which an effect on inter-individual distance and swimming depth was recorded, but with no significant influence of pulse repetition interval on recovery to baseline behavior. Our own study tested a larger range of PRRs, and as predicted, found that groups exposed to INTERMEDIATE and SLOW repetitions took longer to return to baseline inter-individual distance. Additionally, INTERMEDIATE repetitions had the longest lasting impact on group swimming speed, indicative of the high correlations known to exist between these behaviors (Kent *et al.*, 2019). These observations are typical of some commonly described characteristics of habituation, whereby the more frequent a stimulation, the more rapid a depletion in response, provided an asymptote has been reached (Rankin *et al.*, 2009).

In this study, the magnitude of change in response to acoustic stimuli was observed to decrease over time. This reduction was described as an increase in tolerance, an instantaneously demonstrable behavior, rather than habituation (Blumstein, 2016). To unequivocally demonstrate habituation, the same individual must repeatedly be tested over time and exhibit a diminished response (Bejder *et al.*, 2009). Alternative forms of sensory adaptation, such as a hearing threshold shift could also explain the return to baseline behavior (Rankin *et al.*, 2009). While this explanation cannot be ruled out without the support of species-specific data, temporary threshold shifts (TTSs) observed in closely related species [e.g., *Carassius auratus*, Smith *et al.* (2004)] suggest that louder SPL and longer durations of acoustic exposure would be required to induce TTSs in minnow encountering a stimulus with the spectral and temporal attributes of those used in this study. Reductions in behavioral and physiological [e.g., ventilation rate; Nedelec *et al.* (2016)] responses to repeated anthropogenic noise exposure may act as a learned adaptive mechanism, allowing individuals to remain in an affected environment. In the absence of another paired predictive stimuli, or negative reinforcer (e.g., visual presence of a predator), tolerance and habituation are examples of single-stimulus learning, or irrelevant stimuli filtering (Rankin *et al.*, 2009; Blumstein, 2016). While such processes are beneficial in optimising the fitness of individuals reliant on, for instance, site-specific spawning grounds, on-the-other-hand, sustained or cumulative

exposure to stressors is known to have physiological consequences. For example, overall fitness may be impacted in terms of growth, body condition, reproduction, predator-avoidance, or foraging behavior (Nedelec *et al.*, 2015; Sabet *et al.*, 2015). Furthermore, from a conservation perspective, a diminished response to a deployed stimuli could render acoustic guidance systems ineffective at reducing fish injury or mortalities.

This study employed a reductionist, tightly controlled approach using a small tank setup to provide a stable, easily modelled and reproducible acoustic field, in which the influence of confounding factors could be minimized. This approach ensured that the response of freshwater fish to sounds differing only in their temporal characteristics could be fundamentally addressed, prior to implementing any cost-heavy field studies. Sound stimuli generated within such a laboratory setup promote highly complex acoustic conditions, whereby a tank's small size, large impedance, wall material properties (influencing resonance frequencies), and sound speed differences between the water and surrounding air produce high levels of particle motion within the sound field that are understood to differ from large-scale "natural" aquatic environments (e.g., oceans or deep lakes) (Akamatsu *et al.*, 2002). Comparatively, the acoustic nature of rivers, shallow streams (sometimes <1 m depth), or man-made channels where acoustic deterrents may be deployed are not well understood.

Riverine habitats can be exceptionally diverse, and highly engineered banks and channels in combination with other abiotic factors (e.g., wind) create multiple boundary environments that can also influence sound propagation and the pressure/particle velocity relationship. Sound fields within rivers have high reverberation, and are more complex, and less predictable than those of deeper marine or estuarine systems (with shallow water defined up to ∼500 m) (Katsnelson *et al.*, 2012). Acoustic conditions are influenced by fluctuating depths and narrow channels, differing topographies, air entrapment, hydraulic conditions, and temperature and salinity clines (Tonolla *et al.*, 2010). While approaches are being taken to understand common acoustic patterns within physically or ecologically distinct river and shallow stream habitats (Tonolla *et al.*, 2010), the heterogeneous nature of these aquatic systems restricts the quantification of a "typical" natural riverine environment. Field trials to validate the results of our study are therefore highly recommended to confirm the findings in settings where acoustic conditions reflect those that freshwater fish may more typically encounter, in combination with other confounding variables (e.g., seasonal flow) (Tonolla *et al.*, 2010). Additionally, this would allow for the investigation of response depletion to repeated exposures of stimuli over time in an environment where animals have the option to swim away (Popper and Hastings, 2009), subsequently better informing the development of appropriate acoustic deterrent technologies.

The PRRs tested during our study had consistent interval timings between each acoustic exposure or regular pulse rates. Less predictable broadband noise signals using

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irregular pulse repetition intervals have been observed to have greater influence on habituation rate or anxiety reduced responses than regular pulse rate intervals in individual zebrafish [*Danio rerio*: Sabet *et al.* (2015)]. However, these results have yet to be replicated in experiments involving groups of fish, with inconsistent findings in both laboratory [zebrafish: Neo *et al.* (2015a)] and *in situ* field studies [European seabass: Neo *et al.* (2016)]. While it is hypothesized that the influence of group dynamics in these studies obscured any subtle influences of irregular PRR on group behavior (Neo *et al.*, 2016), we found this not to be the case in this study with regular pulse rates.

V. CONCLUSIONS

Our study highlights an effect of temporal variation (PRR) on the group behavior and tolerance of a shoaling freshwater fish species. INTERMEDIATE PRRs were observed to induce a longer lasting shift in group behavior from the baseline, across all behavioral parameters. The study adds to a growing body of evidence that indicates that intermittent sound has a stronger and longer lasting impact on fish behavior than continuous sound differing only in temporal structure. Results are promising for informing the development of more effective and sustainable acoustic deterrent systems. Further investigation in the field is required to validate technologies and better understand the longer-term effects of tolerance or habituation to sounds differing in bandwidth, entropy (e.g., tonal versus broadband), and temporal characteristics (regular/irregular PRR and amplitude ramp-up).

[1]See supplementary material at https://doi.org/10.1121/10.0000910 for exemplar video images used to examine minnow behavioral changes.

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