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RESEARCH ARTICLE

The limits of applicability of the sound exposure level (SEL) metric to temporal threshold shifts (TTS) in beluga whales, *Delphinapterus leucas*

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ABSTRACT

The influence of fatiguing sound level and duration on post-exposure temporary threshold shift (TTS) was investigated in two beluga whales (*Delphinapterus leucas*). The fatiguing sound was half-octave noise with a center frequency of 22.5 kHz. TTS was measured at a test frequency of 32 kHz. Thresholds were measured by recording rhythmic evoked potentials (the envelope following response) to a test series of short (eight cycles) tone pips with a pip rate of 1000 s^{-1}. TTS increased approximately proportionally to the dB measure of both sound pressure (sound pressure level, SPL) and duration of the fatiguing noise, as a product of these two variables. In particular, when the noise parameters varied in a manner that maintained the product of squared sound pressure and time (sound exposure level, SEL, which is equivalent to the overall noise energy) at a constant level, TTS was not constant. Keeping SEL constant, the highest TTS appeared at an intermediate ratio of SPL to sound duration and decreased at both higher and lower ratios. Multiplication (SPL multiplied by log duration) better described the experimental data than an equal-energy (equal SEL) model. The use of SEL as a sole universal metric may result in an implausible assessment of the impact of a fatiguing sound on hearing thresholds in odontocetes, including under-evaluation of potential risks.

KEY WORDS: Whale, Hearing, Noise, Threshold shift

INTRODUCTION

The negative impact of anthropogenic noise on the hearing of cetaceans, particularly odontocetes, attracts much attention. The sensitive and wide-ranging hearing of toothed whales and dolphins, which is used for both passive hearing and echolocation (Au, 1993; Nachtigall et al., 2000), may be particularly susceptible to damage by intensive noise. The impact of noise on this auditory system is known as the permanent or temporary threshold shift (PTS or TTS, respectively), which is a reduction in sensitivity. Understanding the dependence of TTS and PTS on fatiguing sound parameters is of great importance for the establishment of appropriate protective measures and regulations.

Many factors influence the TTS and PTS effects, including noise spectral content, level, exposure duration, time after noise exposure, continuous or intermittent manner of exposure, and subject species. Among these factors, noise level and duration are the most obvious: the higher the level and/or the longer the exposure, the greater the effect. This regularity has been shown in many investigations performed in humans and laboratory mammals (reviews by Miller et al., 1963; Clark, 1991; Melnick, 1991; Yost, 1994). Therefore, it was logical to combine these two parameters to evaluate fatiguing sound efficiency based on the equal energy hypothesis, i.e. to specify the sound exposure in terms of overall sound energy. With this approach, the fatiguing sound is specified in terms of the equivalent sound pressure level (L_{eq}), based on the time–intensity trade of 3 dB per doubling time (ISO R-1999, 1971).

The same approach has been used in TTS investigations in odontocetes. Fatiguing sounds have been characterized by their sound exposure level (SEL), which is a dB measure of the temporal integral of intensity. With the use of squared sound pressure level (SPL) as a metric of intensity, SEL may be specified as dB re. 1 μPa^2 s. When the SPL is kept constant during the exposure, the SEL is simply a dB measure of the product of the squared sound pressure and the exposure duration. This parameter is an equivalent of a dB measure of the overall sound energy flux density (J m^{-2}), which uses the squared sound pressure (Pa^2) instead of the power flux density (W m^{-2}). Along with several other metrics (peak SPL for pulse-like sounds), the SEL metric has been widely used and is recommended to characterize fatiguing sounds (Southall et al., 2007; Finneran and Jenkins, 2012).

The SEL is a convenient metric for characterization of fatiguing sounds when either SPL or exposure duration is kept constant: the higher the SEL, the greater the TTS. However, SEL is not a universal metric when both SPL and duration vary. Restrictions in the use of SEL follow, in particular, from fundamental data obtained in laboratory animals (Carder and Miller, 1972; Clark, 1991). These data have shown that duration-dependent TTS growth depends on the fatiguing sound level, and conversely, level-dependent TTS growth depends on fatiguing sound duration. Thereafter, the time–intensity trade may be either lower or higher than 1 dB dB^{-1}, which contradicts the equal energy hypothesis. Additionally, TTS studies in humans and laboratory mammals have revealed the existence of 'effective quiet', i.e. SPL low enough to produce no TTS even at long exposure. Finally, long exposure to fatiguing sounds produces an asymptotic threshold shift; prolongation of the exposure beyond the asymptotic threshold shift limit does not increase TTS despite further SEL increases (reviewed in Melnick, 1991).

Investigations in odontocetes have revealed patterns that in many respects resemble those found in laboratory mammals. In particular, TTS dependence on the SPL of a fatiguing sound becomes steeper with an increase in the sound duration, and TTS dependence on duration becomes steeper with an increase in the sound SPL. These patterns were summarized in a model in which TTS is the product of the TTS versus SPL and TTS versus log duration functions (Finneran et al., 2010a). This model predicts that the time–intensity

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Received 24 October 2013; Accepted 17 February 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.098814

List of symbols and abbreviations

EFR	envelope following response
k	proportionality factor
L_{eq}	equivalent sound pressure level
PTS	permanent threshold shiftn
R	intensity/duration ratio
SEL	sound exposure level
SPL	sound pressure level
T	duration
TTS	temporary threshold shift

trade becomes either lower or higher than 1 dB dB^{-1}, depending on the intensity/duration ratio.

Indeed, available data have demonstrated different manners of TTS dependence on the intensity/duration ratio of the fatiguing sound. In a study by Nachtigall et al. (Nachtigall et al., 2003), a 50 min bandpass (4 to 11 kHz) noise exposure of 179 dB re. 1 μPa (i.e. an SEL of 214 dB re. 1 μPa^2 s) resulted in a TTS of up to 11 dB, whereas in a study by Finneran et al. (Finneran et al., 2007), a 64 s exposure to a 20 kHz tone of 185–193 dB re. 1 μPa (SEL of 203–210 dB re. 1 μPa^2 s) resulted in a larger TTS of up to 40 dB. Although a quantitative comparison of these two investigations cannot be made because different fatiguing sounds were used, the results suggest a trend of larger TTS at higher levels and shorter exposures than at lower levels and longer exposures of the same SEL. A similar relationship has been found in a study on finless porpoises (*Neophocaena phocaenoides*), demonstrating that a change of SPL resulted in a TTS versus SEL growth of 1 dB dB^{-1}, whereas extension of the noise duration resulted in TTS versus SEL growth of 0.4–0.6 dB dB^{-1} (Popov et al., 2010). Under other conditions, the relationship might be the opposite. Finneran et al. (Finneran et al., 2010a) have demonstrated a substantial increase in TTS with increasing exposure duration while keeping SEL constant. The same observation has been presented by Kastelein et al. (Kastelein et al., 2012): bandpass fatiguing noise centered at 4 kHz resulted in higher TTS at an SPL of 124 dB re. 1 μPa than noise of the same SEL at an SPL of 136 dB re. 1 μPa. Mooney et al. (Mooney et al., 2009) have reported threshold TTS at lower SEL when they used noises of lower SPL and longer duration as compared with higher TTS and shorter duration.

A few independent observations cannot compose an entire picture of the TTS dependence on the intensity/duration ratio or delimit the ranges in which TTS is dependent on or independent of the intensity/duration ratio. For a more complete understanding of this dependence, a more systematic investigation of TTS at various combinations of these two parameters is necessary, which was the goal of the present study. Specifically, we investigated the influence of fatiguing sound level and duration on post-exposure TTS in beluga whales, *Delphinapterus leucas* (Pallas 1776).

RESULTS

Test response features

Rhythmic sound pip trains (Fig. 1) were used as test stimuli for threshold measurements (see Materials and methods). These stimuli provoked a rhythmic version of the auditory evoked response: the envelope following response (EFR), which was a burst of waves of the same frequency as the stimulus pip rate (Fig. 2A). The response was delayed relative to the stimulus by a lag of ~4.5 ms, which is typical of odontocetes' auditory evoked potential latency. This lag indicated that the responses were neurophysiological and not artifacts. The frequency spectrum of the 16 ms window containing the response featured a definite peak at the stimulus rate frequency of 1 kHz (Fig. 2B).

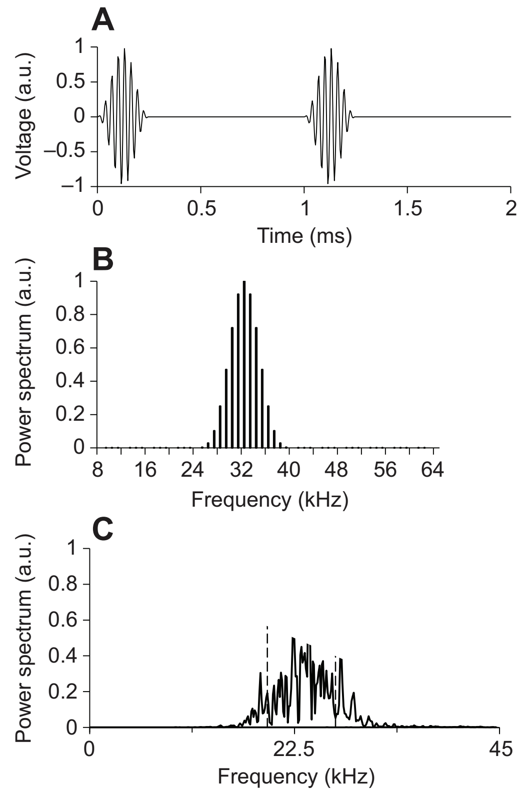

Fig. 1. Waveforms and spectra of the signals. Waveform (A) and spectrum (B) of the probe signal. For better resolution, only an initial segment containing two pips of the 16-pip train is presented in A. (C) Frequency spectrum of fatiguing noise; a spectrum of an 8 s noise sample centered at 22.5 kHz is presented; vertical dashed lines and the double-headed arrow delimit nominal 0.5 octave frequency bands (19.5 to 29 kHz).

The EFR magnitude was level-dependent: with a stimulus level decrease, the magnitude decreased until disappearance. When the stimulus level varied in a near-threshold range according to the adaptive procedure, in the majority of cases, one 5 dB step resulted in the transition from response presence to response absence and back, as exemplified in Fig. 2C for a control no-exposure tracing. According to an adaptive threshold evaluation procedure described in the Materials and methods, all instant thresholds during this part of threshold tracing were evaluated as 52.5 dB re. 1 μPa. In some cases, the transition required a stimulus-level change by two steps (10 dB).

Control and post-exposure threshold tracing and TTS evaluation

Examples of control (no exposure) and post-exposure threshold tracing using the adaptive threshold evaluation procedure are presented in Fig. 3. In the control tracing (1), instant thresholds varied mostly within a range of ±2.5 dB. The trend was negligible and statistically insignificant: –1.4±0.8 dB per log time unit (mean ± s.e.m.) (Fig. 3, trace 1).

Immediately after exposure to fatiguing noise, the threshold increased relative to the pre-exposure threshold; traces then revealed gradual threshold recovery (Fig. 3, traces 2 and 3). The recovery was traced for as long as 1800 s irrespective of whether the recovery was complete (trace 2) or partial (trace 3). TTS was never observed 24 h post-exposure, as shown by pre-exposure measurements for the next-day experiment. During the recovery,

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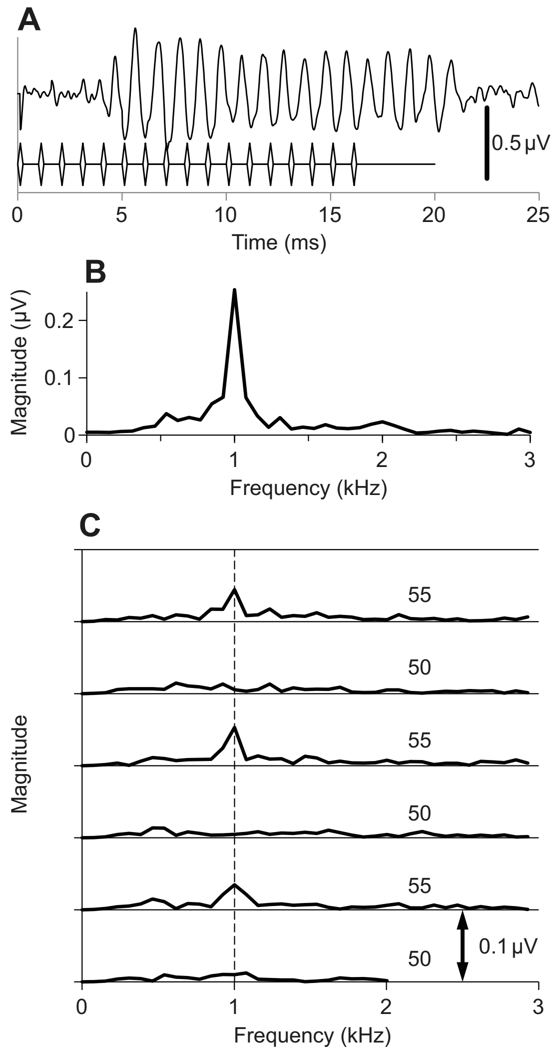

Fig. 2. Threshold determination by recording the envelope following response (EFR) to rhythmic pip trains. (A) Waveform of EFR to a probe stimulus of a sound pressure level (SPL) of 75 dB re. 1 μPa (upper trace) together with the stimulus envelope (lower trace). (B) Frequency spectrum of the record in A. (C) Frequency spectra of EFRs to probe stimuli of SPL varying from 50 to 55 dB re. 1 μPa, as indicated near the records; stimuli of SPL of 55 dB re. 1 μPa produce spectrum peaks at the 1 kHz frequency (marked by vertical dashed line), whereas stimuli of SPL of 50 dB re. 1 μPa produce no peaks.

the threshold versus time functions could be satisfactorily (R^2=0.91 to 0.98) approximated by a log regression line. Trends of the exemplified recovery functions at two exposure durations (3 and 10 min, as indicated) were –21.7±0.9 (R^2=0.95) and –22.8±0.8 dB per log time unit (R^2=0.96; Fig. 3).

Post-exposure thresholds were estimated based on these log regression lines. The value of the regression function 120 s after the fatiguing noise ended was taken as an estimate of the immediate post-exposure threshold. The difference between the threshold estimate obtained in this manner and the pre-exposure threshold was taken as the TTS. This method of TTS evaluation took into consideration not one instant post-exposure threshold, but many points of threshold tracing, making the estimate less dependent on random fluctuations. In the example presented in Fig. 3, the TTS estimates were 25 dB after a 165 dB, 3 min noise exposure and 40 dB after a 165 dB, 10 min exposure.

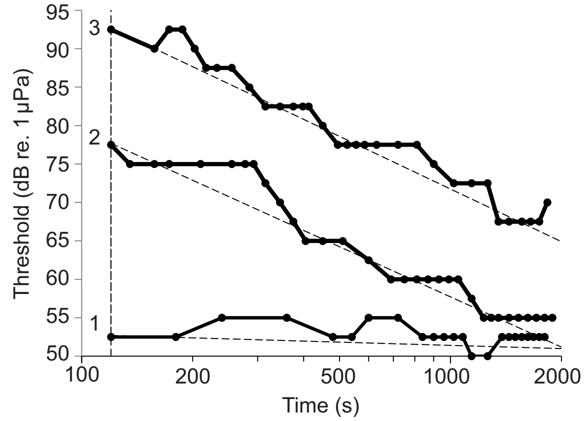

Fig. 3. Post-exposure threshold dynamics. 1, Control dynamics (after no exposure); 2, dynamics after exposure to noise at 165 dB re. 1 μPa, 3 min; 3, dynamics after exposure to noise at 165 dB re. 1 μPa, 10 min. Vertical dashed line marks the 120 s post-exposure time, oblique dashed lines mark the regression lines. Data are from subject 1.

Series 1: TTS dependence on level and duration of fatiguing noise

Series 1 was performed in the female subject. In this series (23 exposures), all combinations of fatiguing noise SPL from 155 to 170 dB re. 1 μPa (by 5 dB increments) and durations from 18 to 6000 s (by 3× increments) were tested. The only exception was a combination of the highest SPL (170 dB) and the longest duration (6000 s), which was not tested for the animal's safety. In total, 23 combinations were tested. TTS was estimated as described above, i.e. by finding the regression slope of the post-exposure threshold versus time function and taking the 120 s post-exposure point of the line as a TTS estimate.

The results demonstrated a monotonic increase of TTS with the increase of both SPL and duration of the fatiguing sound (Fig. 4). The TTS versus SPL dependences were satisfactorily approximated by linear regression lines (R^2=0.80 to 1.0; Fig. 4A). The TTS versus duration dependences were satisfactorily approximated by log regression lines (R^2=0.91 to 0.99; Fig. 4B).

The slope of the TTS versus SPL functions increased with the increase in sound duration (Fig. 4A): from 0.40±0.14 dB dB^{-1} at 18 s duration to 2.75±1.01 dB dB^{-1} at 6000 s duration. All of the TTS versus SPL regression functions, irrespective of the sound duration, crossed the zero TTS value at SPLs from 153 to 155 dB.

Similarly, the slope of the TTS versus log duration functions increased with increasing SPL (Fig. 4B): from 3.0±0.2 dB per log unit at 155 dB SPL to 17.0±1.0 dB per log unit at 170 dB SPL. All of the TTS versus duration regression functions, irrespective of the sound SPL, crossed the zero TTS value at durations from 4 to 18 s).

The same dataset was used to characterize how TTS depended on the intensity/duration ratio of the fatiguing sound at a particular SEL. In Fig. 5, TTS is plotted as a function of the intensity/duration ratio, keeping SEL as a parameter. The intensity/duration ratio was specified by its dB measure R, which is the difference between SPL, a dB measure of intensity, and a dB measure of duration T:

$$R = \mathrm{SPL} - 10\log T\,, \quad (1)$$

where SPL characterizes intensity in dB of squared sound pressure, T is specified in s, and R is specified in dB re. 1 μPa^2 s^{-1}.

With the tested ranges of SPL and duration, the minimum available SEL was 178 dB re. 1 μPa^2 s (from 165 dB, 18 s to 155 dB, 180 s); the maximum available SEL was 203 dB re. 1 μPa^2 s (from

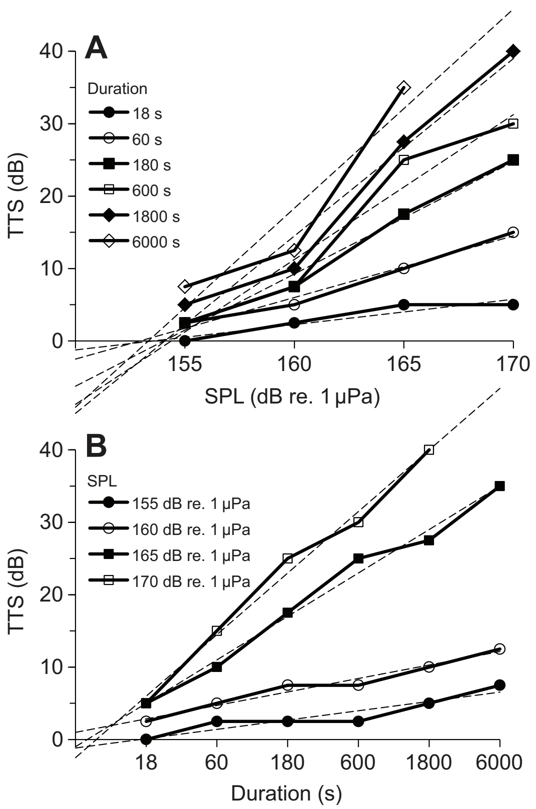

Fig. 4. Temporary threshold shift (TTS) dependence on SPL and duration of fatiguing noise. (A) TTS as a function of SPL, keeping exposure duration as a parameter. (B) The same data as a function of exposure time, keeping SPL as a parameter. Dashed lines indicate regression lines. Data are from subject 1.

180 dB, 1800 s to 165 dB, 6000 s). The intensity/duration index *R* varied from 118 dB re. 1 μPa^2 s^{-1} (155 dB SPL, 6000 s duration) to 158 dB re. 1 μPa^2 s^{-1} (170 dB, 18 s).

Keeping SEL constant did not result in constant TTS when the intensity/duration index varied. At lower *R* (approximately from 118 to 138 dB re. 1 μPa^2 s^{-1}), the TTS versus *R* dependence was positive: more intensive and shorter sounds produced higher TTS than less intensive and longer sounds of the same SEL. At higher *R* (~143 dB re. 1 μPa^2 s^{-1} and above), the tendency was reversed: more intensive and shorter sounds resulted in a lower TTS than less intensive and longer sounds of the same SEL.

The dataset produced by this series did not allow tracing of the TTS versus *R* dependence at high intensity/duration ratios. A tendency toward a negative trend was observed at SELs of 178 to 188 dB re. 1 μPa^2 s; however, at a higher SEL, the functions did not extend far enough along the *R* axis to reveal the further trend. Therefore, in the next series of experiments, the TTS effects were studied using combinations of SPL and duration of fatiguing noise that provided longer constant-SEL TTS versus *R* functions.

Series 2: TTS dependence on the intensity/duration ratio in fatiguing noise

This series was (23 exposures) performed in the male because the female used in the previous series was no longer available. In this series, the SPL and duration of fatiguing noise were varied from session to session to keep constant one of the following SELs: 178 dB re. 1 μPa^2 s (from 175 dB SPL, 2 s to 150 dB, 600 s); 183 dB re. 1 μPa^2 s (from 180 dB SPL, 2 s to 150 dB, 1800 s);

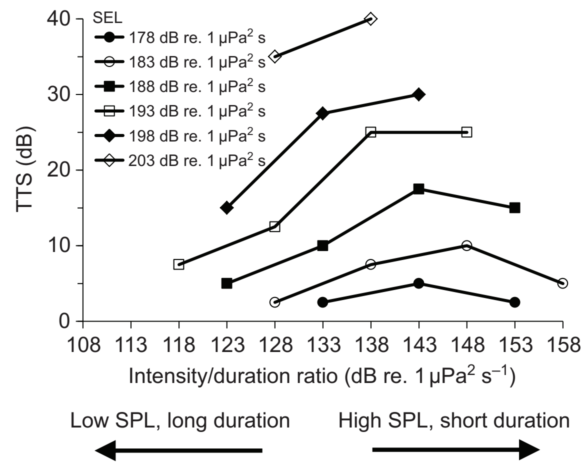

Fig. 5. TTS dependence on the SPL versus duration ratio, keeping sound exposure level (SEL; dB re. 1 μPa^2 s) as a parameter. Data are from subject 1.

188 dB re. 1 μPa^2 s (from 180 dB SPL, 6 s to 155 dB, 1800 s); or 193 dB re. 1 μPa^2 s (from 180 dB SPL, 18 s to 160 dB, 1800 s).

These combinations were not favorable for plotting TTS versus SPL and TTS versus log duration functions such as those shown in Fig. 4 because only two to four points were available for each of those functions. However, these combinations provided six to seven points for each of the SELs except for the highest SEL of 193 dB (four points).

TTS was estimated using the same criterion as in the previous series, i.e. by finding the regression slope of each of the threshold versus time (post-exposure) functions and taking the 120 s post-exposure point on the regression line as a TTS estimate.

The measurements revealed non-monotonic TTS dependence on the intensity/duration ratio (Fig. 6). At lower intensity/duration ratios, the trend was positive: the higher the intensity/duration ratio, keeping SEL constant, the higher the TTS. At high intensity/duration ratios, the trend was the opposite: the higher the intensity/duration ratio, the lower the TTS.

The inflection points from positive to negative trends were in the intensity/duration ratios from 143 dB (at SEL of 178 dB) to 158 dB re. 1 μPa^2 s^{-1} (at SEL of 193 dB re. 1 μPa). The available data did not allow statistical estimation of the variation in the inflection

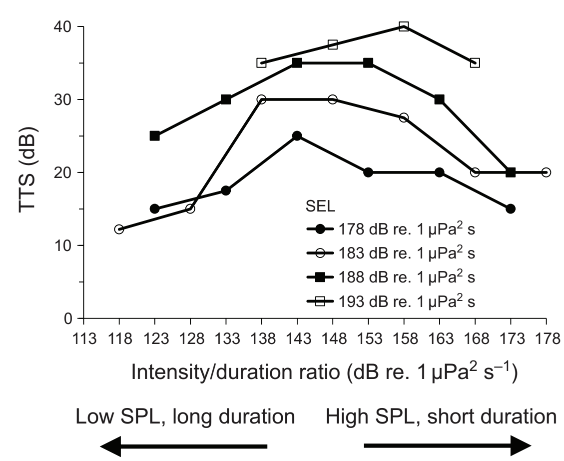

Fig. 6. TTS dependence on the SPL versus duration ratio, keeping SEL (dB re. 1 μPa^2 s) as a parameter. Data are from subject 2.

point position. Nonetheless, the functions at all SELs demonstrated that inflection from a positive to a negative trend appeared within a certain intermediate range of the intensity/duration index.

The TTS dependence on SEL was generally monotonic: keeping the intensity/duration index constant, the higher the SEL, the higher the TTS. In Fig. 6, this tendency manifests itself in the mutual position of the plots: the higher the SEL, the higher the position of the TTS versus R plot. However, higher TTS values were possible at a lower SEL with an effective intensity/duration ratio than at a higher SEL with an ineffective intensity/duration ratio. For example, a SEL of 178 dB re. 1 μPa^2 s and R of 143 dB re. 1 μPa^2 s^{-1} produced a higher TTS (25 dB) than a higher SEL of 183 dB and R of 118 dB re. 1 μPa^2 s^{-1} (12.5 dB) or R of 168–178 dB re. 1 μPa^2 s^{-1} (20 dB). Similarly, an SEL of 183 dB re. 1 μPa^2 s and R of 138–148 dB re. 1 μPa^2 s^{-1} resulted in a higher TTS (30 dB) than did a higher SEL of 188 dB re. 1 μPa^2 s and R of 123 dB re. 1 μPa^2 s^{-1} (25 dB) or 173 dB re. 1 μPa^2 s^{-1} (20 dB).

DISCUSSION

Comparison of the data with multiplication and equal energy models

The data obtained in Series 1 allow comparisons with different models of TTS dependence on fatiguing sound intensity and duration: (1) the equal energy model assumes that TTS is proportional to sound intensity and time, i.e. to the sum of dB measures of intensity (SPL) and duration (time logarithm); and (2) the log multiplication model assumes that TTS is proportional to the product of dB measures of intensity (SPL) and duration (time logarithm).

The equal energy model is represented by the following equation:

$$\mathrm{TTS} = \mathrm{SPL} + 10\log T - \mathrm{SEL}_0\,, \qquad (2)$$

where SPL is intensity in dB re. 1 μPa^2, T is the duration of the sound in s, and SEL_0 is the minimal effective SEL (dB re. 1 μPa^2 s) that determines the TTS values. The log multiplication model was represented by the equation:

$$\mathrm{TTS} = k(\mathrm{SPL} - \mathrm{SPL}_0) \cdot 10(\log T - \log T_0)\,. \qquad (3)$$

where k is a proportionality factor. The two factors in parentheses are excess of SPL and time logarithms above SPL_0 and $\log T_0$, respectively, where SPL_0 and T_0 are the minimal values of SPL and T required to produce any TTS. For both of the models, the values of k, SPL_0 and T_0 were iteratively adjusted until the best fit to the experimental data was achieved according to the least-mean-square criterion.

The result of this procedure (Fig. 7) showed that the equal energy model fits the experimental data (Fig. 7A) worse than does the log multiplication model (Fig. 7B). For the equal energy model, the minimal achieved root-mean-square (r.m.s.) disagreement was 5.8 dB at SEL_0=173.5 dB; for the log multiplication model, the minimal achieved r.m.s. disagreement was 1.7 dB at SPL_0=153.7 dB re. 1 μPa, T_0=8.7 s and k=0.11. The obtained values of SPL_0 and T_0 were within the ranges of scatter of their estimates by regression lines: from 153 to 155 dB (see Fig. 4A) and from 4.0 to 18.0 s (see Fig. 4B), respectively.

Thus, the log multiplication model better describes the TTS dependence on fatiguing noise intensity and duration in the experiments described above.

The simple log multiplication model presented above implies the presence of both minimal sound intensity and minimal duration, below which TTS does not appear. The minimal intensity in the model may correspond to the 'effective quiet'; the modeling indicates this intensity to be ~154 dB re. 1 μPa. As to the minimal

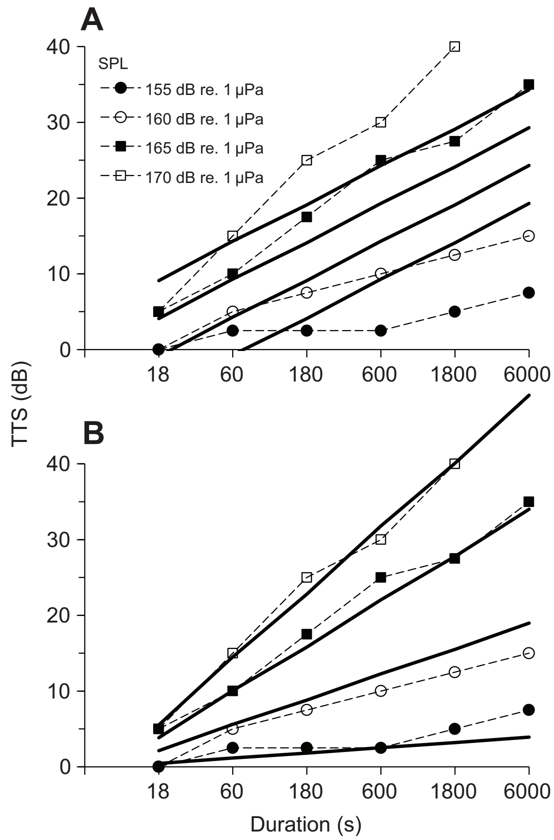

Fig. 7. Approximations of experimental data by different models. Data from subject 1 (see Fig. 4) are approximated by the equal energy (A) and multiplication (B) models. Dashed lines with symbols: experimental data are presented as functions of exposure duration, keeping SPL (dB re. 1 μPa) as a parameter. Bold straight lines: approximations were adjusted according to the least-mean-square criterion.

duration, it is merely a feature of the simple idealized model because experimental data (Finneran et al., 2000; Finneran et al., 2002; Lucke et al., 2009) indicate that short pulses of high intensity are capable of producing TTS. It is reasonable to assume that at short sound durations, their physiological effect cannot be shorter than a certain minimum, even if the physical duration of the sound is very short.

Non-constant TTS at a constant SEL

The log multiplication model implies that TTS cannot be constant at a constant SEL irrespective of the intensity/duration ratio. This property of the log multiplication model is illustrated in Fig. 8, which presents TTS as a product of SPL and the duration logarithm. Parameters for the log multiplication model (see Eqn 3) were rounded to: SPL_0=150 dB re. 1 μPa, T_0=10 s and k=0.1. The diagram shows that at a certain constant SEL, TTS non-monotonically depends on the intensity/duration ratio: it increases with the intensity/duration ratio at low ratios and decreases at high ratios. There is an intensity/duration ratio (in the diagram, it is 140 dB re. 1 μPa^2 s^{-1}) that produces the highest TTS at any particular SEL.

This idealized diagram cannot pretend to exact quantitative simulation of the experimental data presented above, but it simulates the data qualitatively. Presenting the data from both Series 1 and 2 as functions of the intensity/duration ratio, keeping SEL constant, demonstrated TTS variations similar to those predicted by the diagram.

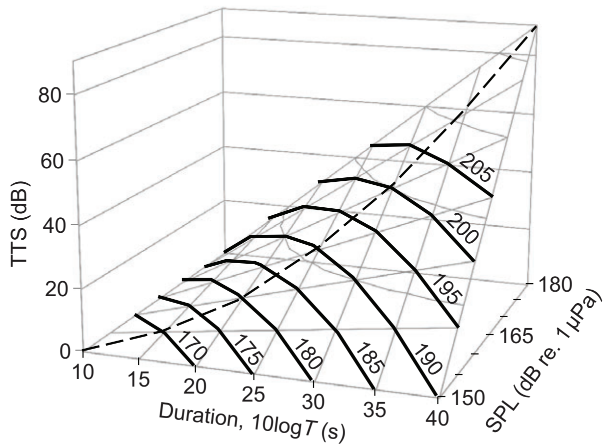

Fig. 8. Modeling of TTS dependence on both SPL and duration of fatiguing sound, using the multiplication model. Bold lines show TTS for various SELs (from 170 to 190 dB re. 1 mPa^2 s, as indicated near the curves) at various combinations of SPL and durations. Dashed line ('ridge' of the 3 day surface) depicts the SPL/duration ratio producing the highest TTS at any SEL.

The variation of TTS depending on the intensity/duration ratio is a substantial deviation from the complete (equal energy) time–intensity trade, which is a theoretical basis of the concepts of both L_{eq} and SEL. If a sound is of high SPL and short duration, a change of the exposure time influences TTS more than the energetically equivalent change of SPL; on the contrary, if a sound is of low SPL and long duration, a change of the SPL influences TTS more than the energetically equivalent change of the exposure time. Therefore, adequate characterization of a fatiguing sound requires a more complicated approach than using SEL alone. Notably, at the most effective intensity/duration ratios, TTS appeared at rather low SELs.

Generalization of the data

A weakness of the present study was that only two subjects were available, with some parts of the study performed in only one of the two subjects. Therefore, generalization of the data to other subjects in the same species or other species is questionable. However, it should be stressed that at least a part of the dependence of TTS on intensity/duration ratio was qualitatively similar in both subjects, and non-constancy of TTS at constant SEL with a varying intensity/duration ratio has been noticed in a few previous studies (see Introduction), even if the TTS dependence on the intensity/duration ratio was not investigated in detail.

Considering all of these data, one may suggest that non-constancy of TTS at constant SEL when the intensity/duration ratio varies is a real event that must be taken into consideration when the effects of fatiguing noise on odontocetes' hearing are assessed. The use of SEL as a sole universal metric, without consideration of the intensity/duration ratio may result in an implausible assessment of the impact of a fatiguing sound on the hearing thresholds in odontocetes. One consequence of this may be an under-evaluation of the potential risks of the noise impact.

MATERIALS AND METHODS

Subjects and facilities

The study was conducted at the Utrish Marine Station of the Russian Academy of Sciences facilities located on the Black Sea coast. The subjects were two young beluga whales, *Delphinapterus leucas*, a 2-year-old male (body length 264 cm, body mass 270 kg) and a 2-year-old female (body length 240 cm, body mass 250 kg). The animals were wild-caught 2 months before the study and adapted to being housed in a pool (9×4×1.2 m) filled with seawater; during these 2 months they did not participate in other TTS studies. The care and use of the animals complied with the Guidelines of the Russian Ministry of Science and Education on the use of animals in biomedical research.

Test and fatiguing sounds

The test sound stimuli were trains of tone pips. Each train was 16 ms long and contained 16 pips at a rate of 1000 pips s^{-1}. Each pip of the train contained eight cycles of a 32 kHz carrier frequency enveloped by a cosine function (Fig. 1A). Thus, the pip duration was 0.25 ms. The frequency spectrum of the pip (Fig. 1B) was 0.25 octave wide at a level of 0.5 of the power peak (–3 dB); its equivalent rectangular bandwidth was 0.32 octave. This type of test stimulus was used because it more effectively produced an EFR than a narrow-band sinusoidally modulated tone (Supin and Popov, 2007). The stimulus bandwidth was sufficiently narrow to assign a resulting measurement to the particular sound frequency with a tolerance of ~0.25 octave. The pip trains were presented at a rate of 16 trains s^{-1}. SPL of the pip trains was specified in dB r.e 1 μPa of r.m.s. sound pressure over the 16 ms pip-train duration.

The fatiguing noise was half-octave band-filtered noise (second-order Butterworth filter) with a center frequency of 22.5 kHz (Fig. 1C). The SPL of the noise was specified in dB re. 1 μPa of r.m.s. sound pressure. The frequencies of the test and fatiguing sounds were chosen because a previous investigation revealed this combination to be very effective at producing TTS in belugas (Popov et al., 2013).

Both the test and fatiguing signals were digitally synthesized at an update rate of 512 kHz by a standard personal computer using a custom-made program (Virtual Instruments) designed with the use of LabVIEW software (National Instruments, Austin, TX, USA). The synthesized signals were digital-to-analog converted by a DAQcard-6062 acquisition board (National Instruments), amplified, attenuated and played through an ITC-1032 (International Transducer Corporation, USA) transducer. The transducer was positioned at a depth of 30 cm, at a distance of 1 m in front of the animal's head, proximal to the front wall of the tank. To amplify and attenuate the test signal, a custom-made amplifier-attenuator of 200 kHz passband was used. To amplify the fatiguing sound, a CV-1800 amplifier (Cervin Vega, USA) of 60 kHz passband was used. The playback channel was calibrated before and after the experiments by positioning a calibrated receiving hydrophone (B&K 8103, Bruel&Kjaer, Denmark) near the animal's head. Sound monitoring revealed that, despite the sound reflections within the tank, local sound levels around the animal's head varied within a range of ±2.5 dB.

Evoked-potential recording and threshold determination

Brain potentials were picked up non-invasively through 15 mm stainless steel surface electrodes mounted within 50 mm silicon suction cups, the active electrode at the vertex of the head surface, 7 cm behind the blowhole and above the water surface, and the reference electrode at the back. Brain potentials were fed through shielded cables to a balanced custom-made brain-potential amplifier based on an AD620 chip (Analog Devices, Norwood, MA, USA) and amplified by 80 dB within a frequency range from 200 to 5000 Hz. The amplified signal was entered into a 12 bit analog-to-digital converter that was one of the A/D channels of the same DAQcard-6062 acquisition board that served for sound generation. The digitized signals were stored and processed on a standard personal computer using a custom-made program (Virtual Instruments) designed with the use of LabVIEW software (National Instruments).

For recording the evoked-potential response, the program extracted 25 ms sweeps from the brain-potential records; the sweeps were synchronous with the test stimuli presentation. To extract the signal from noise, the sweeps were coherently averaged online using triggering from the stimulus onset. For further analysis, a 16 ms segment of the averaged record (the fifth to the 21st millisecond relative to the stimulus onset) containing an EFR to the pip-train stimulus was Fourier transformed online to obtain the response frequency spectrum. With the 16 ms analyzed window, the frequency spectrum resolution was 62.5 Hz. The magnitude of the 1 kHz spectral peak was considered as a measure of the response magnitude.

To trace both the pre- and post-exposure threshold dynamics, an adaptive one-up-one-down (staircase) procedure of stimulus variation was used. To make online decisions concerning the presence or absence of the response, an arbitrary criterion was applied as follows: the program categorized a record as response-present when the 1 kHz peak in the response spectrum was more than twice as high as any of the other spectrum components within the range of 0.75 to 1.25 kHz. Online averaging was continued until either the response-present criterion was achieved or until all spectral components within the range of 0.75 to 1.25 kHz were below 0.01 μV r.m.s.; in the latter case, the trial was categorized by the program as response-absent. Using this rule, 100 to 500 traces were generally averaged to collect one record. With the test signal presentation rate of 16 trains s^{-1}, collection of each record required 6 to 31 s. Stimulus levels were varied by 5 dB increments/decrements. If the response was detected according to the criterion specified above, the next stimulus level was decreased by 5 dB; if the response was absent, the next stimulus level was increased by 5 dB. Reversal points (transitions from stimulus level increase to decrease and vice versa) were identified, and the midpoint of each pair of adjacent reversal points (the local maximum and minimum) was assigned as an instant threshold estimate attributed to the midpoint of the two corresponding time instants.

Experimental procedure, fatiguing noise exposure and threshold tracing

During the experiments, the animals were removed from the home pool and placed on a stretcher in a small wooden tank (4.5×0.85×0.6 m) filled with seawater such that the dorsal surface of the head, including the blowhole, remained above the water surface.

Before the series of noise-exposure experiments, baseline audiogram was collected and control tracing of baseline threshold fluctuations at the test frequency of 32 kHz was performed (see Fig. 3).

Each experiment with fatiguing noise exposure began by re-checking the baseline threshold at the test frequency of 32 kHz. The experiment continued if it did not differ from the baseline threshold by more than ±2.5 dB. The fatiguing noise SPL varied from 150 to 170 dB re. 1 μPa in experiments with the female and 150 to 180 dB re. 1 μPa in experiments with the male. The smaller range of noise levels in experiments with the female was due to lower available output voltage of the power amplifier; in later experiments with the male, the potential of the equipment was extended. Exposure durations varied from 2 to 6000 s. During each experimental session, the fatiguing noise was presented either once or twice. If two exposures were presented during a session, the second exposure was 10 times longer than the first (e.g. 600 s after a 60 s exposure or 1800 s after a 180 s exposure) and was presented 30 min after the previous post-exposure tracing. We assumed this protocol was acceptable because, apart from recovery after the first exposure, the total duration of the two exposures exceeded the second exposure duration by as little as 10%.

Post-exposure thresholds were traced no longer than 1 h, mostly 30 min, even if total recovery was not achieved (see Fig. 3). This limit was applied to restrict the time during which the animal was kept in the tank during the experiment. The longest session time included two noise exposures, two 1 h post-exposure tracings, and a 30 min pause before the second exposure, and was thus ~3 h. With this limit on the total treatment time, the animal lay quietly on the stretcher the entire time it was in the tank, and no disturbances of the animal's behavior were observed upon its return into the home pool.

Acknowledgements
Valuable help of the staff of Utrish Marine Station (Russian Academy of Sciences) is greatly appreciated. Special thanks to Andrey Abramov and Elena Rozanova for the technical and medical support.

Competing interests
The authors declare no competing financial interests.

Author contributions
The study was conceived and designed by V.V.P., A.Ya.S. and V.V.R. Data processing and interpretation, article drafting and revision were carried out by V.V.P. and A.Ya.S. Measurement execution was conducted by D.I.N. and E.V.S.

Funding
The study was supported by The Russian Foundation for Basic Research (grant 12-04-00654) and The Russian Geographic Society (program 'Beluga whale').

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