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RESEARCH ARTICLE

Conditioned hearing sensitivity reduction in a bottlenose dolphin (*Tursiops truncatus*)

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ABSTRACT

The conditioned change in hearing sensitivity during a warning sound preceding a loud sound was investigated in the bottlenose dolphin. Hearing sensitivity was measured using pip-train test stimuli and auditory evoked potential recording. When the test/warning stimulus with a frequency of 22.5 or 32 kHz preceded the loud sound with a frequency of 22.5 kHz and a sound pressure level of 165 dB re. 1 µPa rms, hearing thresholds before the loud sound increased relative to the baseline. The threshold increased up to 15 dB. In order to further investigate whether the observed threshold increase was due to conditioning, the dependence of the effect on warning duration and inter-trial interval was investigated. The duration of the warning substantially influenced the effect. Shorter warnings resulted in deeper suppression of responses and higher threshold increases than longer warnings. In contrast, the effect was nearly independent of the duration of the inter-trial interval, i.e. it was independent of the delay from the loud sound to the test/warning sound in the subsequent trial. These data are considered as evidence that the observed hearing threshold increases were not a result of the unconditioned effect of the loud sound and were instead a manifestation of a conditioned dampening of hearing when the bottlenose dolphin anticipated the quick appearance of a loud sound in the same way as previously demonstrated in the false killer whale.

KEY WORDS: AEP, Damping, Sensation control

INTRODUCTION

The negative impact of loud anthropogenic sounds on whales and dolphins has been demonstrated in a variety of disturbances of their mode of life. In particular, these sounds have been associated with the stranding of whales and dolphins (Evans and England, 2001). Current mitigation procedures to protect whales and dolphins from intense sound focus primarily on finding and avoiding them. Alternative mitigation strategies might be a reasonable augmentation to current efforts, in particular, strategies based on the abilities of the animals to mitigate the sounds themselves. If warned, and allowed to change their hearing sensitivity, dolphins and whales might be able to protect their hearing via self-mitigation. Previous work on terrestrial species has shown that loud sounds can trigger avoidance behavior as effectively as other noxious stimuli (Belluzzi and Grossman, 1969). Escape/avoidance behavioral responses to sounds have also been established in various seal species (Götz and Janik, 2010). One may assume that loud sounds may sometimes be similarly aversive to whales and dolphins and may cause changes in their behavior, allowing animals to avoid loud sounds.

Avoidance responses to loud sounds may manifest themselves in ways other than behavior. Another effect of loud sound is a damping of hearing sensitivity. This damping may be a mechanism of protection from the effects of loud sounds. Several studies have revealed the capability of whales and dolphins to actively control their hearing sensitivity. Measures of the auditory evoked potentials (AEPs) during echolocation have shown that odontocete hearing sensitivity changes to optimize the hearing of echoes (Supin et al., 2005; Supin et al., 2010; Nachtigall and Supin, 2008; Linnenschmidt et al., 2012; Li et al., 2011; Supin and Nachtigall, 2013). Overall the hearing sensitivity of a false killer whale (*Pseudorca crassidens*) was also shown to be more acute when the animal was searching for targets than when targets were easily found (Supin et al., 2008). These findings stimulated a search for the possibility of active damping of hearing sensitivity when a whale or dolphin was warned that a loud sound would soon occur. Indeed, a false killer whale was shown to be capable of damping its own hearing when a loud sound was preceded by a warning faint sound (Nachtigall and Supin, 2013). This in-advance damping may be an effective mechanism of hearing protection and self-mitigation.

The initial demonstration of hearing sensation control, however, left many questions unanswered, requiring additional investigation. First of all, the effect has been demonstrated in only one subject – a single female false killer whale (*P. crassidens*). It would seem reasonable to assess the generalization of the effect with another odontocete species. So, one of the goals of the present study was to check the possibility of the conditioned hearing damping effect in the bottlenose dolphin, *Tursiops truncatus* (Montagu 1821).

A second goal of this study was dictated by the necessity for obtaining more evidence examining the notion that the effect of dampened hearing was due to conditioning and not to some direct (unconditioned) influence of the loud sounds. It is known that the presentation of a loud sound can result in a temporary or permanent reduction of hearing sensitivity, manifesting itself in temporary or permanent threshold shifts (TTS or PTS). These effects have been investigated in both terrestrial mammals (reviewed by Miller et al., 1963; Clark, 1991) and humans (reviewed by Melnick, 1991) and are under investigation in cetaceans (reviewed by Southall et al., 2007; Finneran and Jenkins, 2012). In the present experiments examining conditioning, a loud sound was presented many times during an experimental session. Under these circumstances, a TTS effect could not be excluded by definition because the animal was repeatedly exposed to loud sound. One must be assured that a dampening of hearing like TTS was not causing the effect of sensation change in the conditioning experiments. A regular control to separate a conditioned and a non-conditioned effect would require the presentation of the loud sound without the conditioning (warning) stimuli. However, this control is not possible within the present design because in order to examine the conditioned change

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List of abbreviations

AEP	auditory evoked potential
EFR	envelope following response
i	inter-trial interval
L	long
PTS	permanent threshold shift
rms	root mean square
S	short
SEL	sound exposure level
SPL	sound pressure level
TTS	temporary threshold shift
w	warning time

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of hearing sensitivity, the test stimuli must be regularly presented before the loud sound. The hearing test stimuli must also serve as conditioning signals irrespective of the experimenter's intention to use them as test and warning, or test only, signals.

In order to separate the conditioned and non-conditioned effects on the damping of hearing, a look at a feature of the conditioned effect described in our previous study (Nachtigall and Supin, 2013) can be helpful. The previous false killer whale work demonstrated that the magnitude of the damping of sensitivity before the loud sound was dependent on the delay from the beginning of the warning stimulus to the start of the loud sound. This dependence

would not of course be expected for non-conditioned effects that influence sensitivity after the loud sound. But to be absolutely sure, in the present study, this delay effect was examined in more detail.

Therefore, the tasks of the present study were to: (1) measure the hearing sensitivity before presentation of a loud sound in a bottlenose dolphin, when the loud sound was preceded by a faint warning sound; and (2) measure the hearing sensitivity both at various delays from the warning to the loud sound and at various delays after the loud sound.

In order to measure hearing sensitivity, the AEP method was used because it allowed rapid audiometric measurements (Supin et al., 2001). Several versions of the method allow one to speed up threshold determination. In particular, in order to produce robust rhythmic AEPs known as the envelope following response (EFR), rhythmic trains of short pips may be used as effective test stimuli (Supin and Popov, 2007). In the present study, this method was used for fast sensitivity measurements within a short time of warning before the loud sound.

RESULTS
Evoked potential features and threshold determination

Hearing sensitivity was tested using stimuli presented as trains of short tone (32 kHz) pips (St in Fig. 1A). Each test stimulus train contained 16 pips at a rate of $1000\,\text{s}^{-1}$. While the trains were
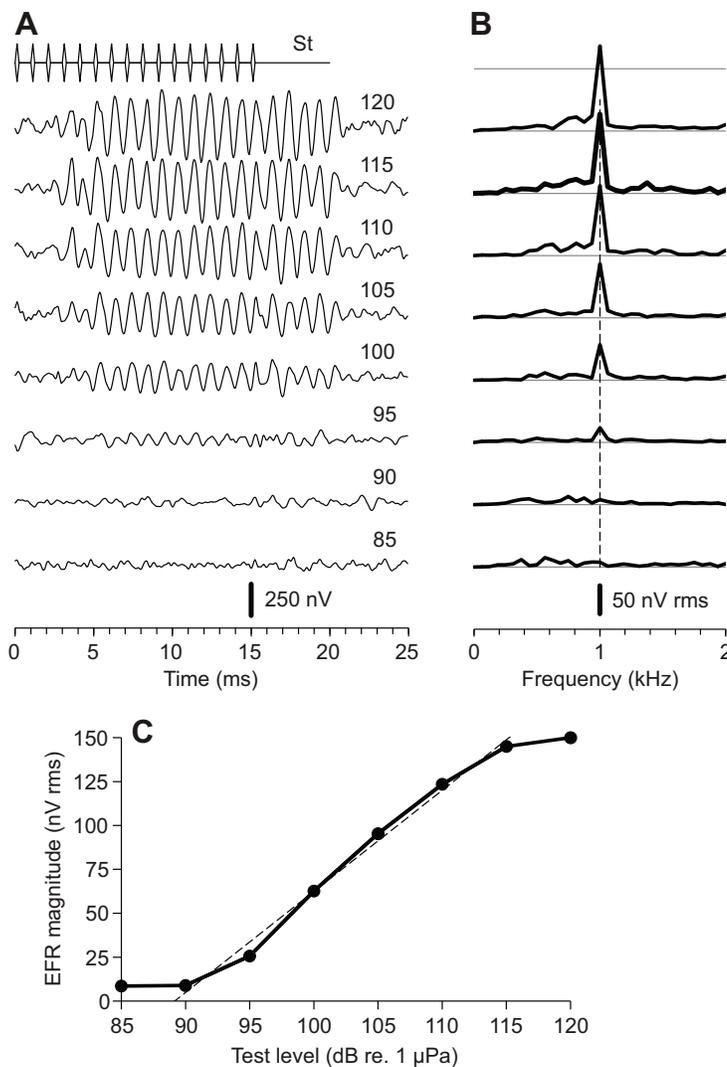

Fig. 1. Example of envelope following response (EFR) records at various test-stimulus levels and threshold determination in a baseline experiment. Test frequency, 32 kHz. (A) EFR waveforms. Test-stimulus levels are indicated near the records in dB rms re. 1 µPa; St, stimulus (pip train) envelope. (B) Frequency spectra of waveforms presented in A. (C) EFR magnitude (1 kHz spectrum peak) dependence on stimulus level. Dashed straight line, regression line approximating a segment of the function from 90 to 115 dB re. 1 µPa rms.

repeated, brain potentials were collected and averaged coherently with the stimuli. The train repetition rate was $15\,s^{-1}$ which allowed data collection fast enough but still did not result in a decrease of EFR magnitude. The averaging revealed a well-defined response as exemplified in Fig. 1A. The response was well depicted as EFRs, i.e. the waveforms featured a series of waves of the same frequency as the rate of tone pips in the test stimulus ($1000\,s^{-1}$). A response lag as long as 3–5 ms relative the stimulus confirmed the neurological origin of the waveforms. The frequency spectra of the records (Fig. 1B) featured a definite peak at the frequency of the stimulation rate of 1 kHz.

In order to assess hearing sensitivity, the signal levels varied from trial to trial in 5 dB steps, obtaining response waveforms at different signal levels, as presented in Fig. 1A. Here and throughout this paper, the signal levels are presented as root mean square (rms) of sound pressure level (SPL) in dB re. 1 μPa. With the signal level decrease, EFR magnitude decreased until it disappeared in the record noise (shown at a level of 90 dB in Fig. 1), as manifested by both EFR waveforms (Fig. 1A) and 1 kHz peak of the record spectra (Fig. 1B). The magnitude of the 1 kHz spectrum peak was taken as a measure of the response magnitude. It was plotted as a function of the test stimulus level, and the oblique part of the plot was approximated by a regression straight line (Fig. 1C). The 'oblique' part of the plot was defined as the part where the slope was not less than 10 nV rms per 5 dB increment, i.e. $2\,nV\,dB^{-1}$ (see Materials and methods). The intersection of the regression line with the zero-magnitude level was used as an estimate of the response threshold. In the presented case (Fig. 1), the threshold was 89.5 dB re. 1 μPa at 32 kHz test frequency.

Using this procedure, baseline thresholds were determined at frequencies of 22.5 and 32 kHz. These frequencies were used for subsequent testing of the damping effect. Each of the averaged responses was gathered over 10 trials, with 75–450 stimuli (pip trains) presentations in each trial, i.e. a total of 160 trials for the set of waveforms at eight stimulus levels and two frequencies (four sessions of 30–50 trials each). The number of stimuli (pip trains) in every trial varied randomly from 75 to 450. Therefore, the number of averaged original records for obtaining each averaged record was not constant and varied from 1275 to 3500. To estimate the influence of the background noise in these records, the same averaging procedure was applied to the control (without stimuli) records with the same number of averaged originals. This resulted in background noise levels of 16–30 nV rms, depending on the average number. The frequency spectra of these averaged waveforms around the target frequency of 1 kHz had background levels of 1–1.8 nV per 62.5 Hz bin.

Based on this data set, the thresholds at 22.5 and 32 kHz were estimated as 84.5 and 89.5 dB re. 1 μPa rms, respectively. These thresholds were roughly 20 dB higher than those typical of the majority of subjects of this species tested by the same method in quiet environments (e.g. Popov et al., 2007) and higher than those in many other odontocete species (reviewed by Nachtigall et al., 2000; Supin et al., 2001); however, they were characteristic of the subject in the present study.

Behavior associated with loud sound exposure

After completion of the baseline series, a series of sessions with presentations of a loud sound in each trial were performed, with 30–50 trials per session. The loud sound was a tone with a frequency of 22.5 kHz, at a level of 165 dB re. 1 μPa and a duration of 5 s. At the first presentation of the loud sound, an element of an avoidance behavior of the subject was observed as a short backward movement, but the animal did not leave its stationing/listening position. This avoidance behavior was extinguished after five or six trials during the first experimental (with loud sound exposures) session. Later on, other disruptive behavior such as head shaking was incidentally observed during the loud sounds, but also without the animal changing its listening position. No avoidance or disruptive behavior was observed during the presentation of faint test sounds before the loud sound. In all the trials, the animal stayed in the listening position until it was called back for fish reinforcement by the trainer.

Manifestation of hearing conditioning effect

In each trial of the conditioning experiments, the faint stimuli (the very same as the test stimuli in the baseline measurements, i.e. the pip trains as described above) were repeated with a rate of $15\,s^{-1}$ during a time randomly varying from 5 to 35 s. As a consequence, 75–525 of these stimuli were presented during each trial. Immediately after the end of these pip-train stimulus presentations, a loud sound followed as described above (a tone of frequency 22.5 kHz, level 165 dB re. 1 μPa and duration 5 s). Thus, in the conditioning series, the pip-train stimuli served both for measuring hearing sensitivity (similar to the baseline series) and for a warning that the loud sound will come shortly after the onset of pip-train stimulation. Therefore, in the conditioning series, these pip-train stimuli preceding the loud one played a double role: test and warning. Therefore, for the conditioning series presented below, they are referred to as test/warning signals.

During a session, 30–50 trials were repeated, each containing the test/warning signal and a following loud sound. Thus, in each trial, the effect of the previous trials was tested during the test/warning time, and presentation of the loud sound reinforced the effect on subsequent trials. Random trial-by-trial variation of the length of the test/warning time from 5 to 35 s excluded the possibility of linking a conditioning effect to a particular time after the warning signal onset.

During the test/warning time, brain responses were collected and averaged in the same manner as described above for baseline records. As the test/warning stimulus durations ranged from 5 to 35 s and stimulus presentation rate was $15\,s^{-1}$, 75–525 original records were gathered in each trial, and data from 10 trials were averaged off-line to obtain the final averaged record. Because of random variation of the test/warning signal duration, the number of finally averaged original records was not equal for all of the final averaged records and the number varied from 1050 to 3500. The background noise of the averaged records was assumed to be the same range as described above based on control (no stimulus) records.

Two frequencies were tested: 22.5 kHz (the same as the loud sound frequency) and a higher frequency of 32 kHz. The obtained EFR waveforms and spectra are exemplified in Fig. 2 for a conditioning experiment when thresholds were measured at a test/warning frequency of 32 kHz (the same as in the baseline experiment exemplified in Fig. 1). The test/warning stimulus levels varied from 85 to 120 dB re. 1 μPa, with inter-trial intervals of 55±5 s.

Fig. 2 demonstrates that at all stimulus levels, the responses were substantially less than at the same levels in the baseline experiment. This manifested itself in both EFR waveforms (Fig. 2A) and spectra (Fig. 2B). The response was absent at stimulus levels of 110 dB re. 1 μPa and below. Only two points (110 and 115 dB re. 1 μPa) fitted the criterion for regression analysis specified above: the slope not less than 10 nV per 5 dB level increment. The line drawn through these two points gave a threshold estimate of 109.0 dB re. 1 μPa, in
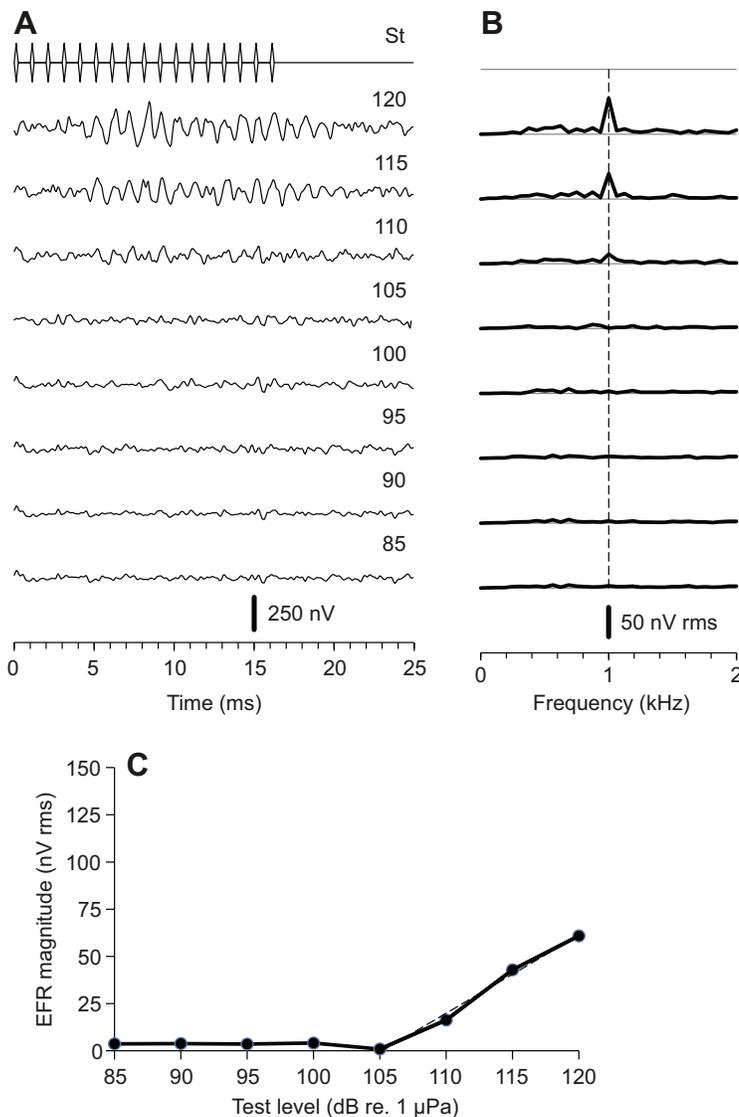
Fig. 2. EFR for an experiment with a loud sound after each test/warning signal. The details are the same as for Fig. 1 but with a 22.5 kHz, 165 dB re. 1 µPa, 5 s sound. Test frequency, 32 kHz; inter-trial intervals, 55. The regression line approximates a segment from 105 to 120 dB re. 1 µPa rms.

contrast to 89.5 dB in the baseline experiments (19.5 dB difference). Note that not only was the threshold increased but also the responses to the suprathreshold stimuli were lower. The suprathreshold stimuli (115–120 dB re. 1 µPa) produced much lower responses than previously shown in the baseline.

Conditioning effect at various warning times and inter-trial intervals

The effects of combining the test/warning and loud sounds were investigated in three configurations or combinations of warning times and inter-trial intervals. (1) Long warning time and long inter-trial intervals. The test/warning time randomly varied from 15 to 75 s (mean 45 s). Because of the long test/warning time, inter-trial intervals in these sessions were kept as long as 105±5 s. (2) Short warning time and short inter-trial intervals. The warning time randomly varied from 5 to 35 s (mean 20 s). The short test/warning time allowed inter-trial intervals as short as 55±5 s. (3) Short warning time and long inter-trial intervals. Similar to configuration 2, the warning time varied from 5 to 35 s (mean 20 s); however, similar to configuration 1, inter-trial intervals were kept as long 105±5 s.

Temporal diagrams of these three configurations are presented in Fig. 3. For brevity, below these three configurations are referred to

as LwLi, SwSi and SwLi, respectively (L, long; S, short; w, warning time; i, inter-trial interval).

These three configurations allowed comparison of the effects of (i) short and long warning times at equal (long) inter-trial intervals and (ii) short and long inter-trial intervals at equal (short) warning times. The comparisons were made at the loud sound frequency of 22.5 kHz and test frequencies of 22.5 and 32 kHz. The configurations were listed successively in the order listed above; two or three everyday sessions for each.

The results of these experiments are presented as plots in Fig. 4. All the combinations of test/warning time and inter-trial intervals resulted in decreased sensitivity compared with the baseline. This effect was more prominent for the test frequency of 32 kHz (shift of EFR magnitude versus level functions of more than 15 dB, Fig. 4A) than for 22.5 kHz (shift of up to 10 dB, Fig. 4B). Considering both test frequencies (22.5 and 32 kHz) and all configurations, an ANOVA revealed significant differences between the baseline and experimental data for all three configurations ($P<0.003$). Although quantitatively different, qualitatively the effects of the warning/test duration and inter-trial intervals were similar for the two test frequencies. Namely, while keeping inter-trial intervals equal (long), the duration of the
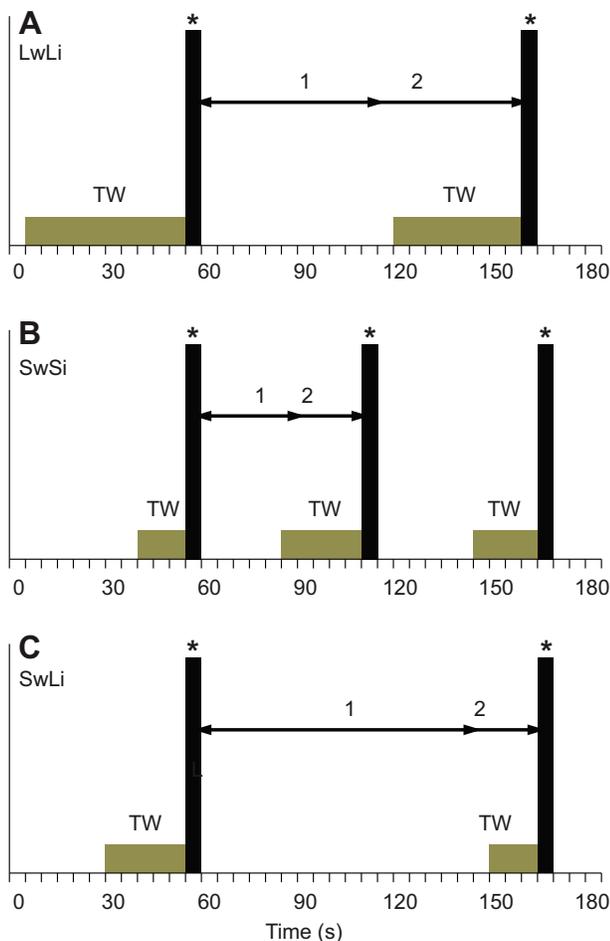
Fig. 3. Temporal diagrams of three experiment configurations with different durations of test/warning signal and different inter-trial intervals. Two (A,C) to three (B) successive trials are presented. The test/warning signal (TW; note variable duration from trial to trial) precedes the loud sound (indicated by an asterisk). L, long; S, short; w, warning time; i, inter-trial interval; 1 and 2, delays from a loud sound to the start and end, respectively, of the test/warning signal in the next trial.

Fig. 4. EFR magnitude dependence on test signal level. Test frequencies of 32 kHz (A) and 22.5 kHz (B) were used in baseline experiments and in experiments with a loud sound after each test/warning signal at different warning durations and inter-trial intervals. LwLi, long (mean 45 s) warning and long (mean 105 s) inter-trial interval; SwSi, short (mean 20 s) warning and short (mean 55 s) inter-trial interval; SwLi, short warning and long inter-trial interval. Dashes straight lines are regression lines approximating oblique segments of the functions.

warning substantially influenced the effect: a short warning resulted in a deeper suppression of responses than a long warning (plots LwLi and SwLi). In contrast, when the warning time was kept constant (short), the effect was nearly independent of the duration of inter-trial intervals (plots SwSi and SwLi).

The same data set was used to assess hearing thresholds at various test/warning times and inter-trial intervals. Thresholds were assessed using the same regression line procedure and the same criterion for selection of points for regression analysis (the slope not less then 10 nV per 5 dB level increment) as described above for the baseline measurements. Note that threshold estimates at 32 kHz SwSi and SwLi are presented without standard errors (s.e.) because s.e. is not applicable for lines drawn through only two points. However ANOVA (see above) showed that the sensitivity shift was significant. The thresholds showed the same regularity as described above. Keeping inter-trial intervals equal (long), the duration of the warning substantially influenced the threshold: short warnings resulted in higher thresholds than long warnings (LwLi and SwLi); keeping the warning time constant (short), the thresholds were only slightly dependent on, or independent of, the duration of inter-trial intervals (SwSi and SwLi) (Fig. 5).
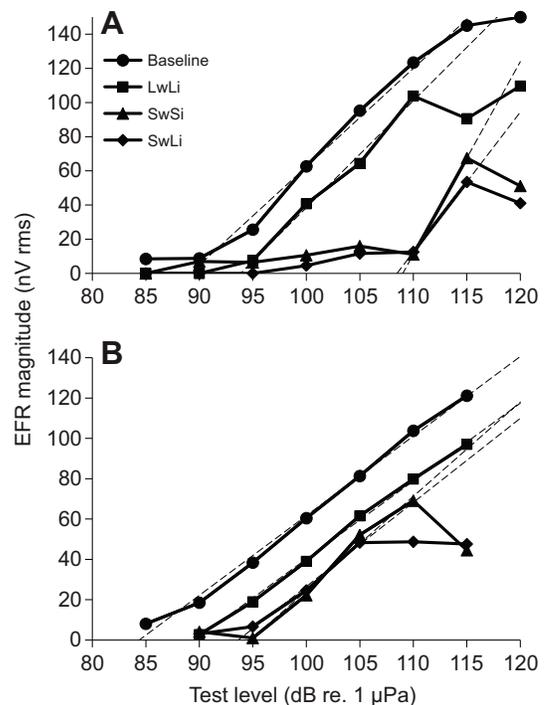
DISCUSSION
Generalization of the data
The results obtained in the present study were similar to those described previously in another odontocete subject of another species (Nachtigall and Supin, 2013). Despite some quantitative differences that may result from differences in the subject species and signal parameters, qualitatively the dampening of hearing when the animal is warned that a loud sound is about to arrive by a preceding faint sound looked similar in the two investigations. This is an argument in favor of the hypothesis that conditioned control of hearing sensitivity is a feature of the odontocete auditory system.

It should be mentioned also that the conditioning effect was described both earlier (Nachtigall and Supin, 2013) and herein at certain arbitrarily used combinations of both test/warning and loud sounds. It cannot be excluded that perhaps with other parameters the effect may be either more or less prominent. Investigation of the influence of all possible variations of signal parameters (sound pattern, frequency, level) could not be performed within the frame of this particular study; however, it may be an important matter of future studies.

Conditioned or non-conditioned effect?
In order to interpret the above-presented data, a crucial question concerns the nature of the observed damping of hearing sensitivity. Did the change in hearing sensitivity appear as a result of conditioning or through some non-conditioned processes? The question arises because presentations of a loud sound may result in unconditioned effects like temporary or permanent decreases of
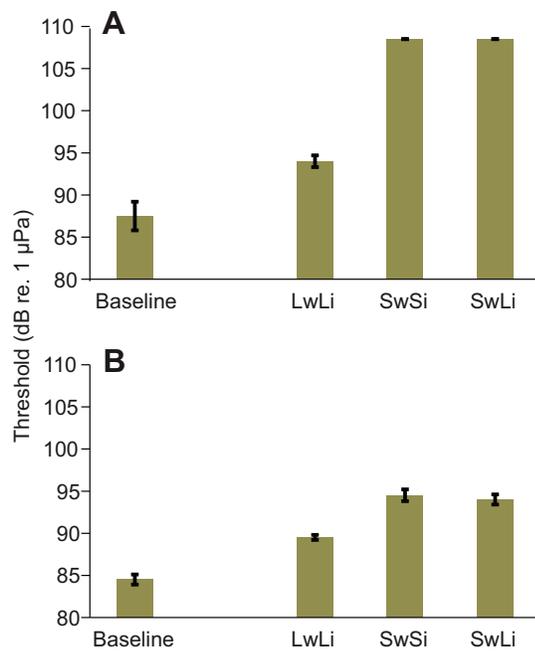
Fig. 5. Thresholds in baseline experiments and in experiments with a loud sound after each test/warning signal at different warning durations and inter-trial intervals. Test frequencies of 32 kHz (A) and 22.5 kHz (B) were used as in Fig. 4. Error bars are standard errors for regression lines crossing the zero level. Standard errors represent deviations of experimental points in the selected segment of the magnitude versus level functions (see Fig. 4) from the straight line (no standard error is shown for SwSi and SwLi at 32 kHz as there were only 2 points).

hearing sensitivity. In the conditioning experiments, trials with the presentation of a loud sound followed one another many times during each experimental session. So in each trial, hearing sensitivity was tested after all of the loud sounds from the preceding trials. In particular, trials occurred shortly (less than 2 min) after the loud sounds in the preceding trial. Within these conditions, neither a short-term TTS effect after the previous loud sound nor a long-term TTS effect due to multiple presentations of the loud sound could be excluded without some sort of examination.

A regular control requiring the presentation of the loud sound without the conditioning stimuli could not be employed here because the test stimuli before each loud sound might have served as warning signals. Moreover, simply sending the animal to the listening position could also serve as a warning signal irrespective of the subthreshold or suprathreshold level of the test/warning sound. But the dependence of the effect on the temporal interrelations between the test/warning and the loud sound indicates that the dampening of hearing was indeed due to conditioning.

A short-term non-conditioned effect (e.g. forward masking) might depend on the delay of the test after the loud sound. It would in no way depend on parameters of the faint test signal before the loud sound. A long-term non-conditioned effect like TTS might depend on the number of presented loud sounds and on how frequently they were presented, but not on parameters of faint tests presented in between the loud sounds. The data presented herein indicate that the damping effect did not depend on inter-trial interval, i.e. on how frequently loud sounds were presented or how long the delay was between the loud sound and the subsequent test. In contrast, the effect did depend on the duration of the warning before the loud sound. Both these features are contradictory to the predictions of a non-conditioned nature of the damping effect.

However, dependence of the effect on the duration of the warning at equal inter-trial intervals is not contradictory to the conditioned nature of the effect because characteristics of the conditioning stimuli may influence the success of conditioning.

The absence of unconditioned effects in the experiments described herein might have been expected because of the rather low sound exposure level (SEL) of the loud sounds. The SEL of each loud sound (165 dB re. 1 μPa, 5 s) was as low as 172 dB re. 1 μPa^2 s. That is much lower than the SEL producing TTS in the majority of studies in odontocetes, e.g. 210 dB re. 1 μPa^2 s (Finneran et al., 2000), 212–214 dB (Nachtigall et al., 2003) and 203–210 dB (Finneran et al., 2007) in the bottlenose dolphin, and more than 183 dB in the finless porpoise and beluga whale (Popov et al., 2011; Popov et al., 2013). So it was not surprising that each of the 172 dB re. 1 μPa^2 s exposures produced no immediately following unconditioned TTS effect. The total exposure during a whole session (30–50 trials) was higher: 187–189 dB re. 1 μPa^2 s. However, this total exposure consisted of short (5 s) exposures separated by long (>50 s) intervals (duty cycles less than 0.1). Such intermittent exposure is much less effective than continuous exposure at producing TTS (Finneran et al., 2010). So the absence of long-term TTS is also expected and easily explained.

It is not clear yet why the short warning time produces a more successful conditioned damping of hearing than a long warning time. It may be hypothesized that odontocetes have an intrinsic motivation to keep their hearing sensitivity as high as possible unless certain circumstances (like the expectation of a loud sound) dictate the opposite. During a long test/warning sound, the long wait for the loud sound may conflict with this motivation. Although the ranges of 'short' and 'long' warning overlapped in our experiments (5–35 s and 15–75 s, respectively), the shorter range of waiting time might be more favorable for conditioning. Further investigations are needed to determine whether this explanation is valid. Independent of the explanation, the fact that the observed damping of hearing sensitivity depends on the duration of the warning supports the hypothesis that this effect is caused by conditioning.

Possible mechanisms of the hearing conditioning effect

A well-known mechanism of regulation of hearing sensitivity is the acoustic, or stapedial, reflex. This reflex manifests itself as a reduction of hearing sensitivity by reflexively tightening the muscles in the middle ear. The stapedial reflex is well known in humans as a response to external loud sounds (Hung and Dallos, 1972) and also serves for hearing protection in echolocating bats in anticipation of self-produced loud sounds (Henson, 1965; Suga and Jen, 1975; Simmons et al., 1992). However, the actual conditioning of the stapedial reflex in bats has not been demonstrated. Actual protection of hearing by using reflexive hearing sensation changes was demonstrated in rabbits by inducing the stapedial reflex by stimulating the contralateral ear with sound just prior to the administration of a loud high-frequency pulse (Counter and Borg, 1993).

Another potential mechanism may be the 'active' mechanism of the inner ear provided by outer hair cells in the organ of Corti. This active mechanism is responsible to a great extent for the high sensitivity of hearing. As outer hair cells have rich efferent innervations, they might mediate the regulatory influence of the central nervous system on hearing sensitivity (Winer, 2005; Guinan, 2006). The possibility of efferent regulation of hearing sensitivity at the cochlear level is well known (Galambos, 1956).

Neither this dolphin study nor the previous false killer whale demonstration of hearing sensation level change with warning

(Nachtigall and Supin, 2013) provides any specific data that clearly explain the processes underlying the hearing sensation change. However, they do demonstrate the possibility that this kind of hearing regulation occurs.

MATERIALS AND METHODS

Subject and experimental facilities

This research was conducted under US National Marine Fisheries Service Office of Protected Species Marine Mammal permit to take protected species for scientific purposes number 16992-00. The 27 year old female bottlenose dolphin, *T. truncatus*, used in our study was born in our breeding colony in Kaneohe Bay Hawaii. Approval of the research protocol was obtained from the University of Hawaii Institutional Animal Care Committee. The study was carried out in facilities of the Hawaii Institute of Marine Biology, Marine Mammal Research Program.

The subject was trained to accept suction-cup electrodes for brain-potential recording, to swim into a hoop listening station and to listen to the sound stimuli. She had a moderate hearing loss manifested as a high-frequency cut-off of at 45 kHz and increased thresholds at 80 to 90 dB re. 1 µPa below this cut-off; however, this degree of hearing loss was considered as still suitable for investigation of basic hearing processes. The subject was housed in a floating pen complex moored off Coconut Island, Kaneohe, Hawaii. Experiments were carried out in a section of the pen complex 8×10 m in size.

Experimental procedure

Each daily experimental session started by calling the subject to the trainer and attaching surface suction cups containing electrodes for brain-potential recording. The 10 m long thin flexible cables connecting the suction cups to the equipment allowed the dolphin to move over all the volume of the experimental pen. After the suction cups had been attached, 30–50 experimental trials were run during an everyday session.

Each trial started by sending the subject to a listening station. The station was a hoop fastened at a depth of 80 cm below the water surface. During stationing, low-level test/warning sounds were played (see below: 'Signal parameters'). During the presentation of the test/warning sounds, brain potentials, specifically EFR evoked by the test stimuli, were recorded. These responses served to measure hearing sensitivity (see below: 'Brain potential acquisition and hearing sensitivity assessment'). In baseline-measurement trials, only these test sounds were presented. In experimental (conditioning) trials, a high-level 165 dB re. 1 µPa 5 s (loud) sound was played immediately after the test/warning sound. After the end of the loud sound, a secondary reinforcing whistle was blown and the subject was called back from the listening station and received fish reinforcement.

Signal parameters

The test/warning signals were rhythmic trains of tone pips, each train being 16 ms long. Each train contained 16 pips at a rate of $1000\,s^{-1}$ (Fig. 6A). Each pip contained eight cycles of a carrier frequency (Fig. 6B). From trial to trial, levels of the test signals varied up and down from 80 to 120 dB re. 1 µPa rms. Variation of the test signal level was not adaptive (not staircase), i.e. the level did not depend on the response presence or absence in the previous trial. Irrespective of the response presence or absence, all the 80–120 dB range was examined to obtain information on the response magnitude at both threshold and supra-threshold levels. The loud sound was a tone of 22.5 kHz frequency.

Both the test/warning and loud sounds were digitally synthesized by a standard personal computer using a custom-written program (virtual instrument) designed with the use of LabVIEW software (National Instruments, Austin, TX, USA). The synthesized signal waveforms were played at an update rate of 256 kHz through a 16-bit digital-to-analog converter of a USB-6251 acquisition board (National Instruments). The test/warning signals were amplified by a custom-made power amplifier (passband of 1–150 kHz), attenuated by a custom-made low-noise resistor attenuator, and played through an ITC-1032 piezoceramic transducer (International Transducer Corporation, Santa Barbara, CA, USA) positioned
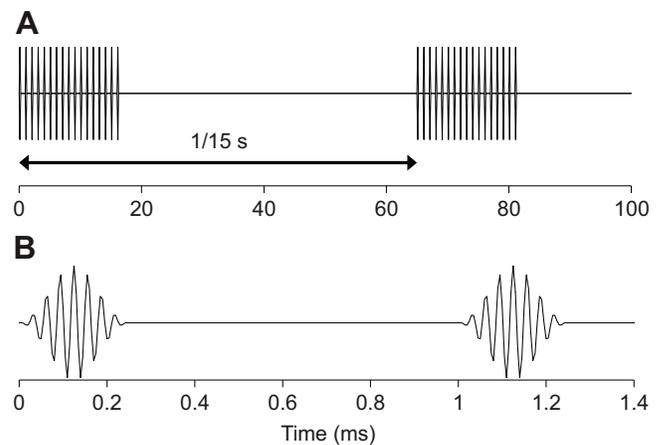

Fig. 6. Waveforms of test stimuli at different time scales. (A) Compressed time scale; two successive pip trains are presented. (B) Extended time scale; two successive pip trains of 16 pips per train are presented.

at a depth of 80 cm (i.e. the same depth as the hoop listening station center) at a distance of 1 m in front of the animal's head.

Signals for the loud sound were amplified by a Hafler P3000 power amplifier (Hafler, Tempe, AZ, USA) and played through the same transducer. The transducer was connected alternately either to the test/warning sound attenuator or to the loud sound power amplifier through an electromagnetic relay, so the background noise of the power amplifier output never overlapped the low-voltage (down to 1 mV) test signals. The re-connection was done simultaneously with the loud sound onset, to avoid any cue preceding the loud sound.

Both test/warning and loud sounds were calibrated by a B&K 8103 hydrophone (Bruel & Kjaer, Naerum, Denmark) positioned in the hoop station in the absence of the subject.

Brain-potential acquisition and hearing sensitivity assessment

Brain potentials were picked up through 10 mm gold-plated surface electrodes mounted within 50 mm silicon suction cups, with the active electrode at the vertex and the reference electrode at the dorsal fin. 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 60 dB within a frequency range from 200 to 5000 Hz. The amplified signal was entered into a 16-bit analog-to-digital converter, which was one of the A/D channels of the same NI USB-6251 acquisition board that served for sound generation. The digitized signals (16 kHz sampling rate) were processed in a standard personal computer using a custom-written program (virtual instrument) designed with the use of LabVIEW software.

The hearing sensitivity assessment was based on recording the EFR to the test tone pips. The brain potentials were averaged on-line within every trial. EFR records obtained by on-line averaging were sorted according to the stimulus frequency and level and were additionally averaged off-line among the trials to obtain final low-noise EFR records. A 16 ms long part of the record, from 5th to 21th ms, containing the EFR was Fourier transformed to obtain its frequency spectrum. The spectrum peak magnitude at the stimulation rate (1000 Hz) was taken as the EFR magnitude. The EFR magnitudes evaluated in this way were plotted as a function of test-signal level. An oblique part of the function was approximated by a straight regression line. This 'oblique' part of the function was defined as a part with point-to-point gradients not less than 10 nV per 5 dB level increment ($2\,nV\,dB^{-1}$). This arbitrary criterion was chosen as allowing separation of the level-dependent segment of the voltage versus level function from its flat parts presenting the background noise at subthreshold stimulus levels and 'saturation' range at high stimulus levels. The point of interception of the regression line with the zero response magnitude level was taken as the threshold estimate (Supin and Popov, 2007).

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