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RESEARCH ARTICLE

Three auditory brainstem response (ABR) methods tested and compared in two anuran species

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ABSTRACT

Hearing sensitivity has been extensively investigated, often by measuring the auditory brainstem response (ABR). ABR measurements are relatively non-invasive, easy to reproduce, and allow the assessment of sensitivity when psychophysical data are difficult to obtain. However, the experimental methods differ greatly in respect to stimulation, which may result in different audiograms. We used three different methods in the same individual frogs: stimulating with brief tone bursts (tABR), long-duration tones (ltABR) and masked ABR (mABR), where transients are masked by a long-duration sinusoid, and the sensitivity is assessed by the difference between unmasked and masked ABR. We measured sensitivity in a range from 100 to 3500 Hz, and the resulting audiograms show two sensitivity peaks at 400–600 Hz and 1500–1600 Hz (both sensitive down to 30 dB re. 20 µPa). We found similar results below 1000 Hz, but when stimulating with long-duration tones, the sensitivity decreased more rapidly above this frequency. We showed that the frequency specificity of tone bursts becomes poorly defined with shorter duration at low frequencies. Comparisons between subjectively (visual inspection by researchers) and objectively (thresholds defined by signal-to-noise ratio) defined audiograms showed very little variation. In conclusion, the mABR method gave the most sensitive audiograms. The tABR method showed a similar audiogram when using relatively long-duration tone bursts (25 ms). The ltABR method is not a good choice for studying hearing thresholds above 1000 Hz because of the bias introduced by spike rate saturation in the nerve fibers and their inability to phase lock.

KEY WORDS: ABR, Auditory brainstem response, Amphibian, Hearing, Hearing sensitivity

INTRODUCTION

Measurement of auditory evoked responses is widely used as a relatively non-invasive method to assess hearing sensitivity. The method has been used on a variety of animals, both vertebrates and invertebrates (Brandt et al., 2018; Brittan-Powell et al., 2002; Christensen et al., 2015a; Corwin et al., 1982; Ladich and Fay, 2013) and amphibians are no exception (Goutte et al., 2017; Schrode et al., 2014; Womack et al., 2017). The response represents the summated activity of the auditory pathway in response to sound stimulation. Usually, the short-latency response (latency <30 ms), termed the auditory brainstem response (ABR), is used to measure thresholds and generate audiograms. These responses are in the microvolt range when measured with dermal or subdermal electrodes, and thus the signal to noise ratio (SNR) affects the measured thresholds. Unlike the ABR of humans in whom the fifth peak is the most prominent of the ABR (Burkard, 2007), the most prominent peak in most animals is the first peak (few milliseconds latency), assumed to be the compound action potential of the auditory nerve. Generally, ABR audiograms have a similar shape to behavioral audiograms in species where both have been measured (Brittan-Powell et al., 2002; Maxwell et al., 2016; Taylor et al., 2019), although the thresholds are usually lower in behavioral audiograms. Thus, ABR measurements allow the assessment of sensitivity in animals that are difficult to condition and where behavioral audiograms therefore would be difficult to obtain.

One general problem in ABR measurements is that the threshold depends on the signal-to-noise ratio (SNR) of the recording, so a low SNR will translate into low sensitivity. SNR depends not only on auditory sensitivity, but also on the spatial configuration of the auditory pathway and distance from electrodes to neural tissue. This may make comparison between species with different skull geometries difficult. Another problem with comparisons between studies is that different ABR methods may result in different shapes of the audiograms. Finally, the way ABR data are analyzed – either by visual inspection or by more objective methods – may conceivably result in differences in the resulting audiograms. This paper aims to investigate and evaluate three different stimulus protocols, in an effort to aid others in choosing the method best suited for their studies.

One of the most common methods used to measure audiograms from ABRs is tone burst stimulation (abbreviated here as tABR) and has been used for measuring hearing in many vertebrate species (Brittan-Powell et al., 2002; Buerkle et al., 2014; Egner and Mann, 2005; Martin et al., 2012; Mooney et al., 2010). Short tone bursts of 5–25 ms are used to stimulate the auditory pathway of the experimental animal to evoke responses and determine auditory thresholds and from these thresholds create an audiogram. However, the use of short bursts is problematic at low frequencies, since the frequency specificity of the stimulus is poor at low frequencies. For example, if stimulating with a nominal 200 Hz sinusoid 5 ms tone burst, only one cycle of the sinusoid is presented (Fig. 1B, time signals), and hence the frequency spectrum contains a wide band of frequencies centered at 200 Hz (Fig. 1B, frequency spectra). Furthermore, to avoid onset clicks, the envelope is shaped by adding rise and fall shaping of the tone burst (usually 2–5 ms duration rise and fall), and thus the already poorly specified frequency becomes more broad-banded because of what is known as 'frequency splatter' (Fig. 1, orange lines are unshaped signals, blue lines are shaped signals). One solution is to adjust the duration of the stimulus tone to always include at least a set number of cycles, e.g. 6 cycles (Brittan-Powell et al., 2010), but this creates another problem, since low frequency stimulation would be of relatively long duration (120 ms at

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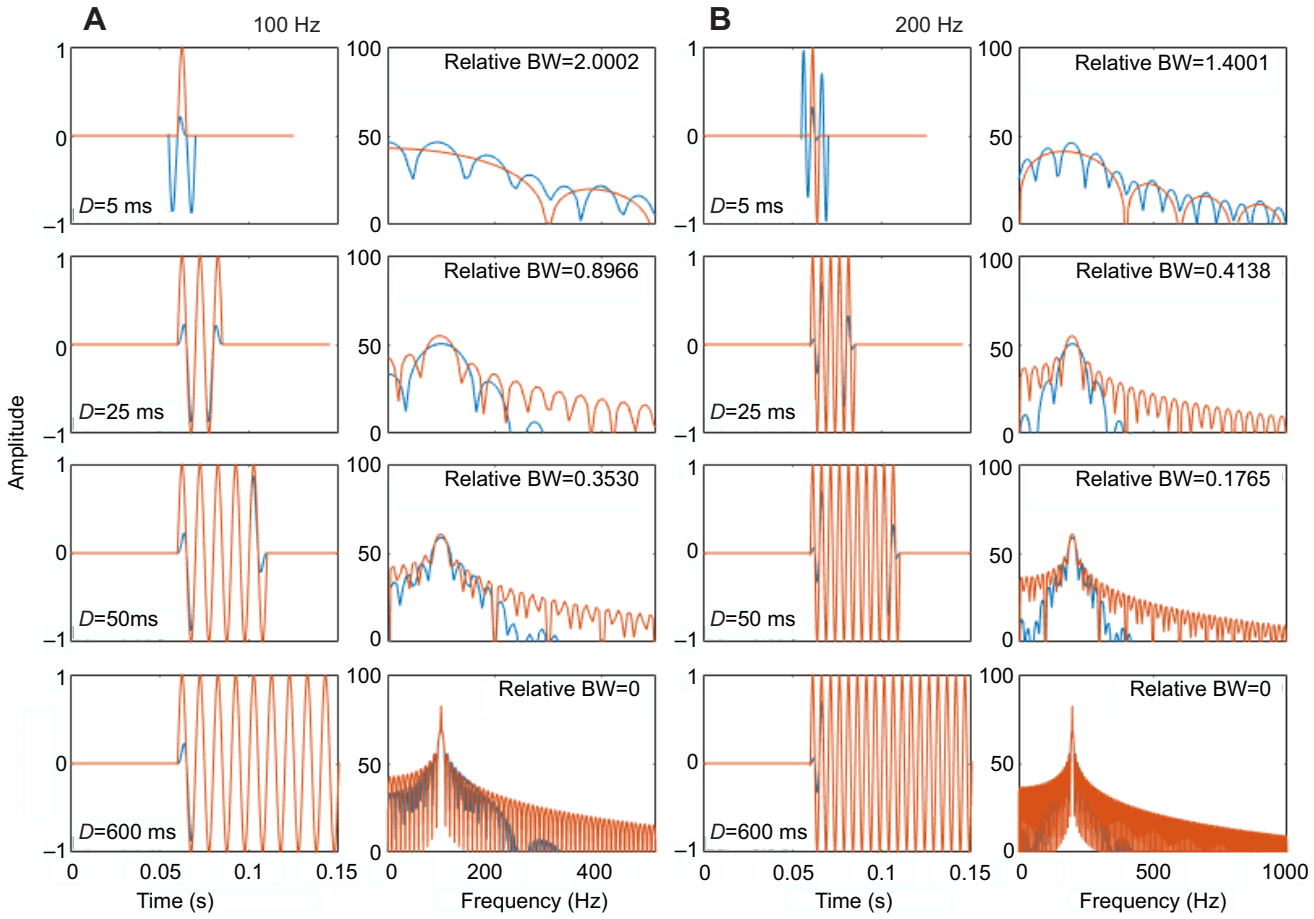

Fig. 1. 100 Hz and 200 Hz tone bursts and their respective frequency spectra. (A) 100 Hz and (B) 200 Hz tone bursts (left) and frequency spectra (right). The orange curves are unshaped tone bursts, the blue curves are shaped signals ($\cos^2$ rise/fall, 5 ms rise/fall time). The relative bandwidth (relative BW) is calculated as the bandwidth of the peak at 10 dB below the peak, divided by the stimulus frequency. *D*, duration of the sinusoid, including rise/fall time.

50 Hz) compared with high-frequency stimulation. In any case it is necessary to find a compromise between duration (and hence the number of cycles) and stimulus presentation, since the steepness of onset envelope influences the generation of ABRs (the steeper the onset, the greater efficacy of ABR generation) (Kenyon et al., 1998). In this study, tone bursts of 25 ms were used, in part to reduce the problems of frequency splatter and how this affects the stimulus, especially at low frequencies. However, most papers use even shorter tone bursts of 5 ms (Baugh et al., 2019; Brittan-Powell et al., 2002; Buerkle et al., 2014).

Frequency specificity of the stimulus can be improved by using long-duration pure tones (from 50 to 600 ms) (Christensen et al., 2015a,b; Kraus and Nicol, 2009; Martin et al., 2012; Piniak et al., 2016), referred to in this paper as long tone ABR (ltABR). The responses from ltABR are evaluated using the frequency spectrum and thus depend on the representation of the waveform in the neural discharges, i.e. on-phase locking to the stimulus tone in the auditory pathway. The response is found at twice the frequency of the stimulus, and this is probably generated by the innervation of oppositely oriented groups of hair cells.

Masked ABR (mABR) (Berlin et al., 1991; Brandt et al., 2007, 2018; Christensen-Dalsgaard et al., 2012) is a relatively new method, based on evaluation of the difference between the response to a brief transient (a click) and the response to a brief transient masked by a pure tone. The click and masked click stimulation are presented alternately, and the sensitivity to the tonal masker is measured as its efficiency in masking the transient. The response is an onset response to the click that does not depend on phase locking in the auditory nerve, and long-duration masker tones can be used to avoid frequency splatter of the stimulus without creating a frequency bias. Another advantage of this method is that it utilizes the relatively strong onset response to the transient stimulus to evaluate ABR and therefore the signal-to-noise-ratio (SNR) is increased. And lastly, by using the transient stimulus, the animal anesthesia level is continuously monitored during the experiment by the unmasked click response.

The aim of the present project is to compare measurements using the three stimulus protocols in the same individuals. Two species of frogs were used, as ABR responses generally are strong and robust in anuran amphibians and we used the same physical setup with subdermal needle electrodes for all measurements. The responses were scored using both objective analyses, based on the signal to noise ratio of the recordings, and subjective evaluation by four researchers, by visual inspection of the responses.

MATERIALS AND METHODS

Experimental animals

This study was conducted on eight adult (individuals A–H) green frogs (*Pelophylax esculentus* Linnaeus 1758) and four adult (individuals I–L) European common frogs (*Rana temporaria* Linnaeus 1758). All animals were wild caught in Southern Funen, Denmark, and they were kept under laboratory conditions in

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paludariums on a 13 h:11 h light:dark cycle at 22°C and with a room humidity of 55%. The experiments were approved by the Danish National Animal Experimentation Board (Dyreforsøgstilsynet).

Anesthesia

Prior to experiments each frog was anesthetized by brief immersion in 2% MS222. The frogs were closely monitored while immersed and level of anesthesia was tested by toe-pinching. When there was no response to toe pinching, the frogs were lightly rinsed in running tap water and placed on a wet towel in the setup. If the frogs started moving during experiments, they were re-immersed in the MS222 solution to deepen anesthesia.

Experimental setup

Auditory brainstem responses (ABRs) were recorded using three electrodes (disposable subdermal needle electrodes, 27 gauge, 12 mm, Rochester Electro-Medical Inc., FL, USA) placed subcutaneously: (1) inverting – dorsal to the ear facing the loud speaker; (2) non-inverting – above the brainstem; and (3) a ground, in the far front leg. The signals from the electrodes were passed through a headstage (TDT RA4LI) and preamplifier (TDT RA4PA) connected to a digital signal processor (TDT RM2) that was controlled by a PC using customized software (‘QuickABR 11th edition’ by C.B.; available upon request). Sound stimulation was controlled by the same software and generated in the RM2, amplified (Cambridge Audio, azur 740A Integrated Amplifier, London, UK) and emitted from a loudspeaker (Wharfedale Diamond 220, Wharfedale Ltd., Huntingdon, UK). The frog was placed inside an audiometric cabin (T-cabin, CA TEGNÉR, Bromma, Sweden) on a table built from consecutive layers of flagstone and mineral wool to minimize noise/vibrations from the floor. The walls inside the cabin were padded with sound absorbing wedge tiles (Classic Wedge 30, EQ Acoustics, UK) and the loudspeaker was placed at an angle to the walls to minimize sound reflections. The loudspeaker was suspended in elastic ribbons to minimize vibration coupling (between the loudspeaker and the animal) via the substrate and was placed approximately 50 cm from the frogs left ear. The setup was calibrated using a ½” microphone (G.R.A.S. microphone type 26AK, G.R.A.S., Holte, Denmark), placed 2 cm above the head of the frog, powered by a microphone amplifier (G.R.A.S. Power Module Type 12AA). The microphone itself was calibrated using a Brüel & Kjær Acoustical Calibrator (type 4321, output: 1000 Hz at 94 dB re. 20 µPa).

Data acquisition

The ABR was recorded for each animal using the three methods: (1) tone bursts (25 ms, $\cos^2$-gated with 5 ms rise/fall time, 200 averages), (2) long tones (600 ms, 40 averages), and (3) masked clicks [clicks (a half cycle of a 4000 Hz sine wave, 0.125 ms duration) alternately unmasked and masked by tones, 320 ms tone on/320 ms tone off, eight clicks in each section, 400 averages] (Fig. 2).

In all three methods, frequencies ranging from 100 to 3500 Hz were tested at different intensities depending on the immediate sensitivity shown by the animal during the trial. When using the tABR and mABR methods, a sequence of lower sound level (in steps of 5 dB) was added to the trial in cases of clear ABRs, and conversely, if no ABR was detected, a sequence of higher sound levels was tested. However, as the custom software only visualizes time domain responses during recording and near-threshold ltABR responses are only visible in the spectrum, it was not possible to add ltABR stimulus based on the responses. Therefore, the intensities tested for the ltABR method relied on experience from the other methods. The system could not generate stimulus levels above 110 dB re. 20 µPa (at some frequencies the highest level was 100 dB re. 20 µPa), and at some frequencies this meant that an ABR could not be measured. All three methods were tested in the same animal on the same day with randomized presentation order of method and frequencies, to counter bias due to fluctuating levels of anesthesia. Fig. 3 shows examples of measurements using the three methods in the same animal and at the same frequency (800 Hz). Note that the mABR signal is the difference signal between the masked and unmasked response.

Data analysis

All data were analyzed both objectively (using custom written scripts) and subjectively by visual inspection by four researchers. The objective thresholds for mABR and tABR were determined by first calculating the signal to noise ratio (SNR) for the response at each stimulus level. Then an exponential function was fitted to the SNR points, and the sound level at the intersection of this curve with a fixed SNR threshold value was the threshold estimate. The objective analysis on ltABR searched for signal peaks at the double stimulus frequency (±10 Hz) while creating a running average of maximum noise in bands of 500 Hz as the noise estimate, calculating SNRs and fitting the values by an exponential function, as above. For the subjective analysis each researcher evaluated all the raw data by inspecting signals as the examples in Fig. 3, and visually determining the thresholds for each frequency as the lowest sound level where a response could be observed, using both peaks and the across-level pattern of the peaks.

The median thresholds, used for creating audiograms, were determined as the median of thresholds of the individuals of both species (*P. esculentus*: 8 individuals, *R. temporaria*: 4 individuals) at specific frequencies of each method. Maximum *n* of subjective methods for *R. temporaria* and *P. esculentus* was 32 and 16,

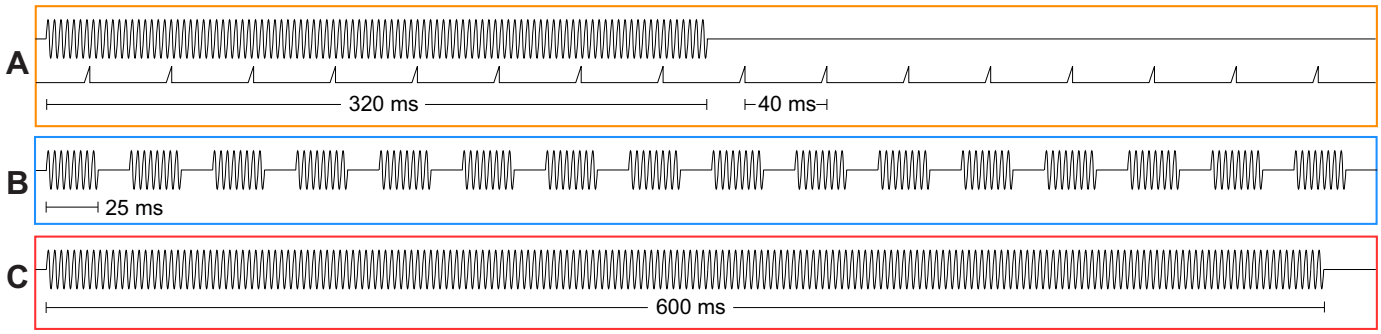

Fig. 2. Diagram of sound stimulation spectra. (A) Masked auditory brainstem response (mABR). (B) Brief tone bursts (tABR). (C) Long-duration tones (ltABR).

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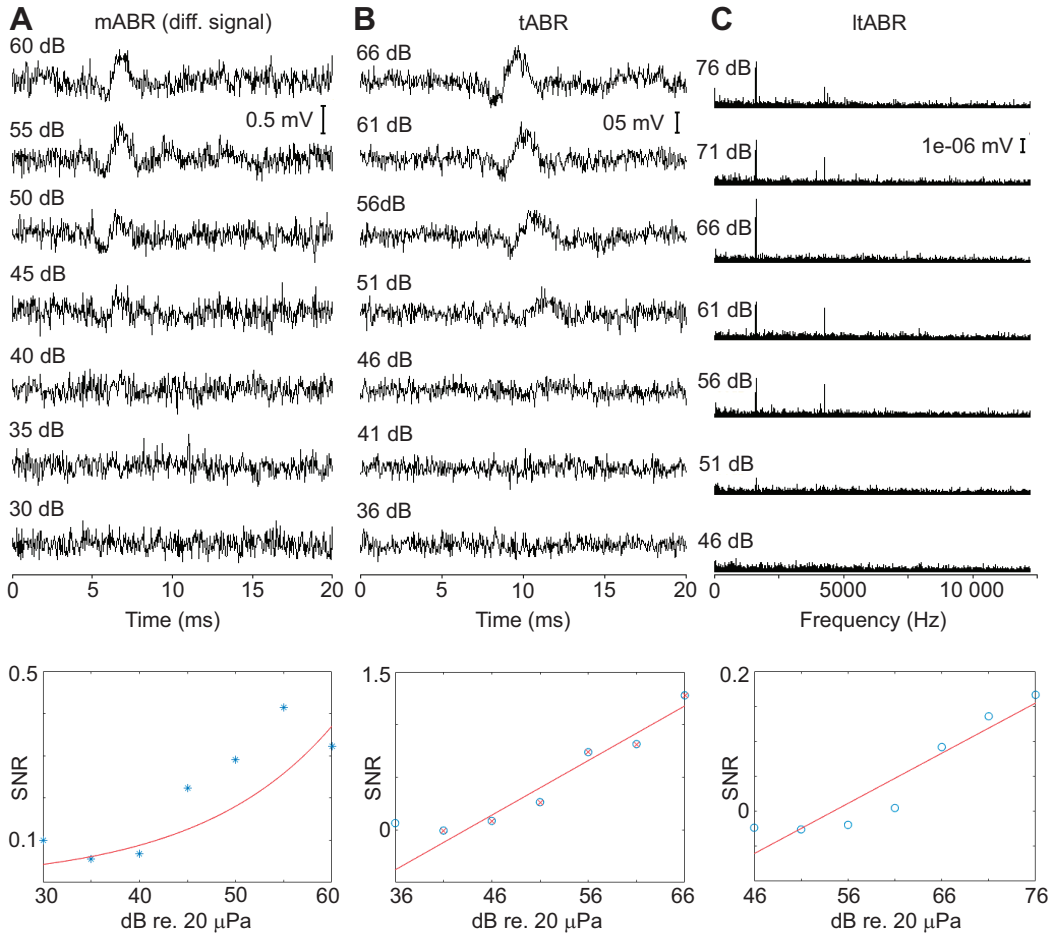

Fig. 3. Raw data for each method and its corresponding SNR for a green frog (*Pelophylax esculentus*) stimulated at 800 Hz. Upper three figures are raw data, used for subjective analysis of mABR (A, the difference signal between masked and unmasked clicks), tABR (B) and ltABR (C). For mABR and tABR methods, the figures show the recorded potential as a function of time. For the ltABR method, the amplitude spectrum of the response is shown. The estimated threshold for mABR was 40 dB re. 20 µPa, tABR was 51 dB and ltABR was 51 dB. Lower three figures show the objective analysis of the same data. The red line is the fitted curve. The intercept of this curve at the given signal-to-noise ratios (SNRs) (mABR: 0.1, tABR: 0.01, and ltABR: 0.0018) estimates the thresholds for mABR at 41.8 dB, tABR at 44.5 dB and ltABR at 63.7 dB.

respectively (number of frogs multiplied by number of researchers). For objective methods n=8 and 4, respectively. The responses to masked stimuli from individual B and C were removed before data analysis due to poor quality of the recordings.

Since thresholds could not be measured at all frequencies owing to the limited range of the sound-delivering system, we also measured the absolute peak values of the responses. These values were determined in two steps: firstly, the time frame (mABR and tABR) of the response peak was determined by visual inspection and the frequency frame (ltABR) was set to 2× stimulus frequency ±10 Hz. Then, secondly, the peak height was measured. For methods mABR and tABR the peak values were determined at 60 dB re. 20 µPa and for ltABR the peaks were determined at 90 dB re. 20 µPa. These thresholds were chosen based on data availability for the respective methods. In files with no responses, noise was measured in the same time/frequency frame as the responses found in other files. The peaks for each animal were normalized to the maximum peak for that specific animal, and then the median for each method was determined.

Statistical analysis

The distribution of thresholds in each method was tested using basic histograms and Shapiro–Wilk test (Shapiro and Wilk, 1965). The variance of the data was tested using an F-test. For comparison of thresholds between the three methods a standard two-tailed paired Student's t-test (α=0.05) was used. Data and statistical analysis were done in R version 3.6.2 and MATLAB version 2019b.

RESULTS

Differences in auditory sensitivity between three methods, mABR, tABR and ltABR, were tested in 12 individual frogs [eight green frogs (*P. esculentus*) and four European common frogs (*R. temporaria*)] at frequencies ranging from 100 to 3500 Hz with intensities from 20 to 110 dB re. 20 µPa. All thresholds were determined both subjectively by four researchers (see Fig. S1 for raw data) and objectively using custom-written scripts. For the ltABRs, no objective thresholds were found at 2000, 2500, 3000 and 3500 Hz for *R. temporaria*, and no thresholds for the latter two frequencies in the *P. esculentus*. All other methods, both objective and subjective found at least one threshold in at least one frog at each frequency. The audiograms were generally V-shaped, but ltABR-derived audiograms had a steeper increase in thresholds towards the higher frequencies. In *P. esculentus*, the subjectively derived ltABR threshold at 3000 Hz was 23 dB above both the mABR and the tABR method, at 2000 Hz it was 22 dB (mABR) and 15 dB (tABR) above, and at 800 Hz the thresholds were 10 dB (mABR) and 3 dB (tABR) above. The same trend of higher ltABR thresholds was seen

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in the objectively derived audiograms, except at 400 Hz, where the ltABR threshold drops below those of the other methods.

The audiograms of *P. esculentus* derived from mABR and tABR were flatter, almost U-shaped compared with the other audiograms, both the ltABR audiogram of this species but also the audiograms of *R. temporaria*. For mABR, the subjectively and objectively determined median thresholds were almost identical, while the two other methods (tABR and ltABR) had some differences between the subjectively and objectively derived medians (Fig. 4). Generally, the mABR method measured lower thresholds than the two other methods. All methods measured almost the same thresholds at 800 Hz and differs the most at higher frequencies.

According to histograms and Shapiro–Wilk tests of data points in each of the three methods, the data were normally distributed, and a *F*-test showed equal variance between the datasets. A two-tailed paired Student's *t*-test (α=0.05) was used to compare the medians of thresholds of the methods pairwise. This test found that the audiograms of each method were significantly different from one another. When comparing the thresholds of mABR with those of tABR (mABR−tABR, t_{14}=−5.516, P=0.05) and ltABR (mABR−ltABR, t_{13}=−3.641, P=0.05) we found that the mABR thresholds were lower than both, and when comparing tABR with ltABR (tABR−ltABR, t_{13}=−3.636, P=0.05), the tABR method generated lower thresholds.

The objective threshold determination depends on the SNR threshold chosen for the analysis. To test the effect of changing the SNR, the responses of one green frog were analyzed at five SNR threshold levels: 1, 0.5, 0.1, 0.05 and 0.01. These audiograms were then compared visually, both comparing them with the subjectively determined thresholds and more importantly, with the shape of the audiogram, to select the SNR best fitted for further analysis on the remaining individuals. The objective analysis on tABR showed that SNRs of 0.0–0.1 result in very similar auditory thresholds, and thus SNRs of 0.01 were accepted for this method. For mABRs, an SNR of 0.1 gives the best fit to the subjective thresholds (Fig. 5).

The normalized peak values for each method are shown in Fig. 6. The peaks have been normalized by dividing the peak amplitude of the ABR response at each frequency by the global maximal peak amplitude for each animal (normalized amplitude=amplitude/max. amplitude). Then, a median of all animals for each method was calculated and plotted with a 95% confidence interval. This was done to facilitate comparison between methods at high frequencies where thresholds could not be determined for the ltABR method. Both tABR and mABR showed responses above 2000 Hz, but for ltABR, method peaks above 2000 Hz were virtually non-existent.

DISCUSSION

We compared auditory thresholds derived from three ABR stimulus protocols; masked stimuli (mABR), tone burst stimulation (tABR) and long tone stimulation (ltABR), and we tested all three methods in the same individual frogs. The resulting audiograms had very similar thresholds at approximately 800 Hz, which is also where the

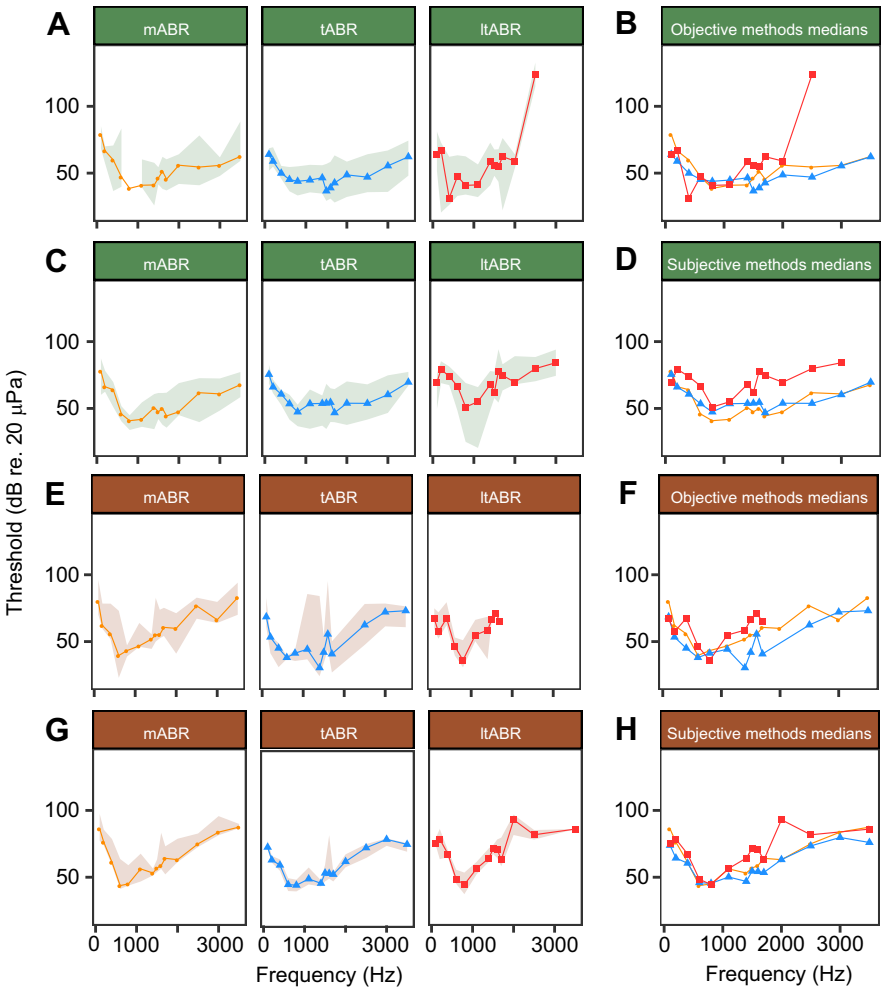

Fig. 4. Objectively and subjectively determined audiograms for the three methods. (A–D) Audiograms from *Pelophylax esculentus* (*n*=8). (E–H) Audiograms from *Rana temporaria* (*n*=4). Each method is designated by its own shape and colour; mABR (●), tABR (▲) and ltABR (■). A and E are medians of the objective analysis method for each ABR method. The ribbon represents the 95% confidence interval. B and F show Comparison of medians of the objective methods. C and G are medians of the subjective analysis method for each ABR method. The ribbon represents the 95% confidence interval. D and H show comparison of medians of the subjective methods.

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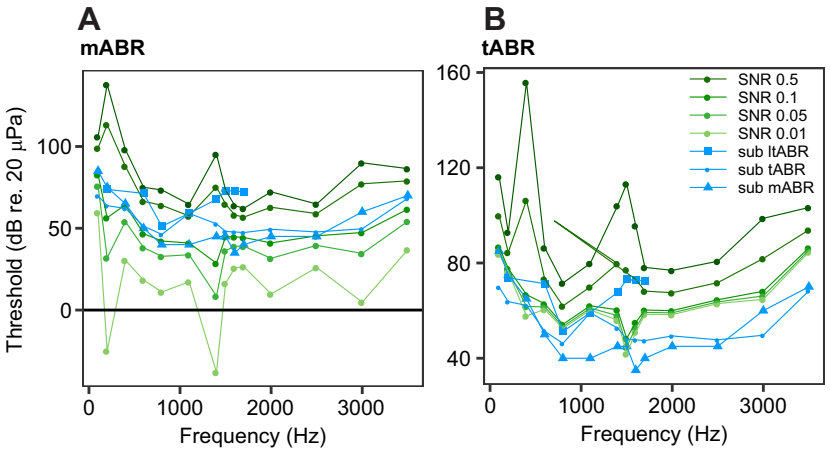

Fig. 5. Audiograms of a green frog (*P. esculentus*) based on objective evaluation of signal-to-noise ratios for mABR and tABR. Audiograms based on objective evaluation of SNRs (calculated at 7–15 ms of the response) at levels 1, 0.5, 0.1, 0.05, and 0.01 (green lines and circle shapes) for (A) mABR and (B) tABR. Blue lines represent the audiograms of subjective methods, mABR (●), tABR (▲), and ltABR (■).

peak sensitivity is also found in both species. This sensitivity peak probably originates in the amphibian papilla. There is a less prominent sensitivity peak at frequencies between 100 and 2000 Hz, which likely originate in the basilar papilla. The mating call of the European common frog has fundamental frequency maximum ranges from 350 to 500 Hz (Brzoska et al., 1977), and the green frogs' call is very broadband, with highest intensity ∼2000 Hz (see Fig. S1).

As shown in Fig. 7, the audiograms of all three methods show comparable thresholds to median thresholds of single auditory nerve fibers in the European common frog (Christensen-Dalsgaard et al., 1998), except for a low-frequency region at 200–400 Hz. However, the thresholds of the most sensitive auditory fibers are 20–30 dB lower. The most notable difference in the audiograms is that the ltABR shows higher thresholds above approximately 1 kHz. We think that this is due to a low-frequency bias in the method, because the ltABR method depends on phase locking in the auditory nerve response. The ability of the auditory nerve fibers to phase lock is primarily limited by the membrane properties of the hair cells, and it is a general observation in all vertebrates, that phase locking declines smoothly with frequency. There is not a definitive cutoff frequency for phase-locking, but in amphibians, phase-locking progressively declines above 1 kHz (Elepfandt et al., 2000; Narins and Hillery, 1983; Schmitz et al., 1992), in goldfish also at ∼1 kHz (Fay, 1978) and in tokay geckos at ∼600–800 Hz (Eatock and Manley, 1981). Additionally, the nerve fibers are limited in their discharge rate and are therefore not able to fire at every cycle at higher frequencies (maximal spike rate reported for frog auditory nerve fibers is 200–300 Hz; Christensen-Dalsgaard et al., 1998; Narins, 1987). Both these sensory and neural limitations will generate a weaker representation of the analyzed frequency and introduce biases for thresholds measured at higher frequencies. Thus, the resulting audiogram will be skewed, compared with audiograms measured using other methods. One problem in comparing thresholds between methods, especially with the ltABR method is that data points at the highest frequencies are missing because the thresholds exceed the stimulus levels that we were able to generate, or possibly because the auditory system is just not sensitive to those frequencies. Here, we investigated the absolute peak values as well, and it is very clear that above 2000 Hz, there is no longer any response when using the ltABR method (Fig. 6), probably because phase-locking has deteriorated, so the signal is completely masked by noise. It is surprising that there are responses at all between 1000 and 2000 Hz, since earlier studies (Elepfandt et al., 2000; Narins and Hillery, 1983; Schmitz et al., 1992) have shown that the ability to phase lock in frog auditory nerve fibers rapidly declines above 1000 Hz.

An additional bias could result from a decline of the response during the long-duration tone stimulation. We tested this by measuring the amplitude at three fixed points in time for all responses to intensities above 75 dB re. 20 µPa (up to 110 dB re. 20 µPa at some frequencies) in individual A (green frog) and found that the amplitude did not change with time (data not shown). So generally, the three methods perform equally well at frequencies below 1 kHz in our setup.

The mABR and tABR methods produce very similar audiograms. One drawback of the mABR method is that the responses are a result of two noisy signals that are subtracted from one another (the masked and unmasked responses, respectively) which decreases the SNR, as shown by comparison of the traces in Fig. 6. This may result in a threshold that is higher than when using tABR. However, we would expect mABR audiogram shapes to be more accurate at low frequencies, where the tone burst method is problematic because of frequency splatter. By stimulating with 25 ms $\cos^2$

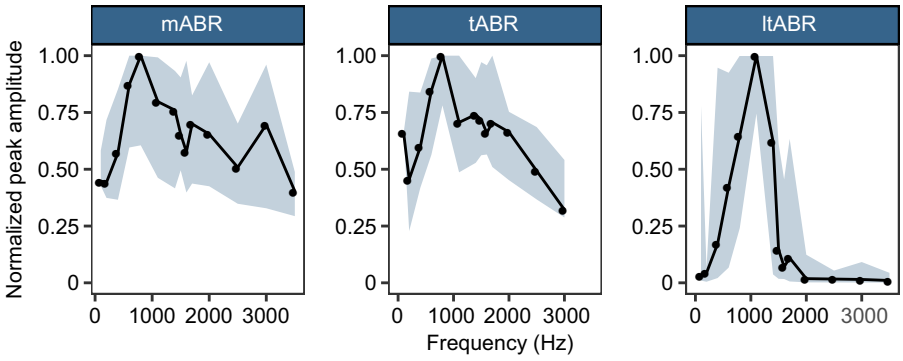

Fig. 6. Normalized peak values of responses from mABR, tABR and ltABR methods. Amplitude of each frequency normalized to the max peak amplitude for each individual for responses from mABR (60 dB re. 20 µPa), tABR (60 dB re. 20 µPa) and ltABR (90 dB re. 20 µPa) methods, respectively. The curves are medians, the grey area shows 95% confidence intervals.

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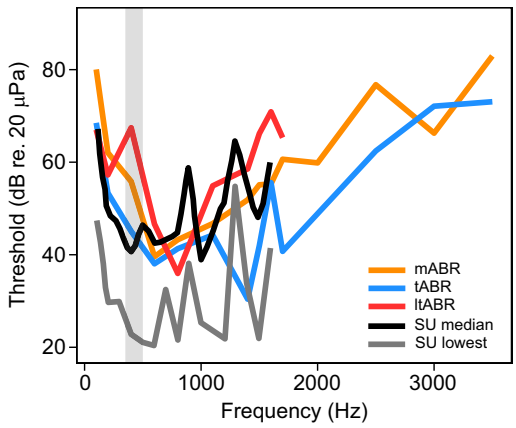

Fig. 7. Subjectively derived audiograms from the European common frog (*Rana temporaria*). Audiograms are compared with single unit thresholds of the auditory fibers in the same species, redrawn from (Christensen-Dalsgaard et al., 1998). Black line is the median and grey line is the lowest measured thresholds. Grey column indicates the call frequency of this species (Brzoska et al., 1977).

shaped tone bursts at 100 Hz, the stimulus is fairly broad-banded, with a relative bandwidth of almost 1 (Fig. 1). Thus, the measured threshold at 100 Hz will reflect the slope of the 'true audiogram' from 50 to 50 Hz, and if this slope is very steep, the low-frequency slope of the tABR audiogram might be different (probably less steep). The differences between mABR and tABR audiograms are also relatively small at low frequencies, but tABR audiograms do show higher sensitivity at 100–200 Hz, which may be due to frequency splatter. That the difference is small is probably due to the relatively long-duration tone burst used (25 ms). It would be interesting to compare our findings with thresholds based on 5 ms tone bursts, since the frequency specificity would be even poorer at low frequencies with shorter tone bursts, as shown in Fig. 1.

All thresholds were determined using both objective and subjective methods (Fig. 8). Subjective methods, i.e. visual inspection of the signals, are often used, especially in hearing clinics, but only a few studies on the inter-observer differences have been made. One study (Vidler and Parker, 2004) shows great differences between audiograms determined by 16 professional audiological scientists or technicians. In this study, two observers had 13 years of experience with the custom-written software (QuickABR, 11th edition), one observer had 5 years of experience and the last observer had one year of experience. All observers were trained in the same lab, with the same experimental setup and the same software, and this is probably why the subjectively derived audiograms are so similar (Fig. S2). One observer retested the subjective method 2 weeks after determining thresholds the first time and found very little test–retest variation. Generally, the objective analysis found lower thresholds than the subjective analysis, and the shapes of the audiograms were also very similar between the two methods. It may be time consuming to develop the scripts needed, but once they are running, the data analysis is faster than using the subjective methods.

Some of the thresholds at specific frequencies vary with up to 30 dB between individuals. It is unlikely that this variation reflects a real sensitivity difference in the population, and the differences measured may be an artifact of fluctuating anesthesia levels. To counter this bias, the order of methods and their test frequencies was randomized, that is, all frequencies for one method were completed before testing the next method. The sequence of methods was randomized between each animal. It would probably have worked even better, had it been possible to randomize both the frequencies and method, but due to constraints in the software, this was not possible. Another solution would be to run the experiments with immobilized animals, instead of anesthetized animals, but that is not allowed by our protocol. By using the mABR method, the anesthesia level is constantly monitored, since the click responses are a very good indicator of the level of anesthesia. If the click responses decline, it indicates that the animal is going into deep anesthesia.

In conclusion, we have shown that all three methods, when tested in the same animal, give similar thresholds below 800 Hz, but not

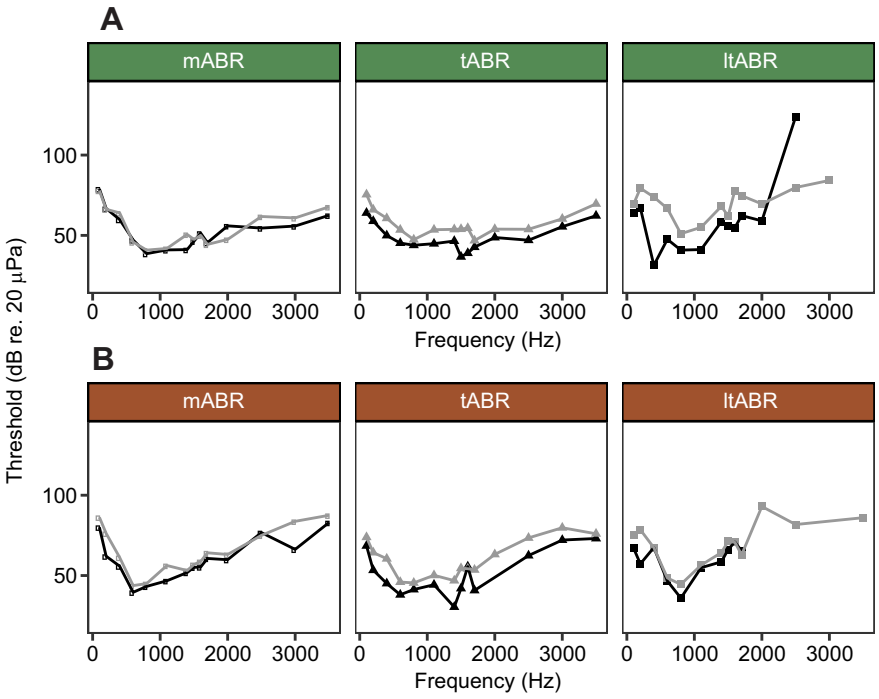

Fig. 8. Comparison of objective and subjective analysis methods for each of the ABR methods in the two frog species. Objective analysis methods (black shapes and line) and subjective analysis methods (grey shapes and line) in (A) *P. esculentus* and (B) *R. temporaria* for each of the ABR methods.

above this frequency. Long tone-derived ABRs (ltABR) are unsuitable for threshold determination above 1000 Hz, since the audiogram becomes skewed, compared with audiograms from the other two methods. We expected the thresholds of the tABR method at low frequencies to differ from the other methods, because of frequency splatter of the stimulus. The difference is small, with the relatively long-duration tone bursts in the present experiment but would most likely be an issue with shorter duration tone bursts. The mABR method showed the most sensitive results, even though thresholds were evaluated using two noisy signals, instead of one as in the other methods. As for the difference between objective and subjective methods we found that the thresholds were almost identical, and this gives hope for faster and more efficient data analysis in the future.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.C.-D., T.B.L.; Methodology: C.B., J.C.-D., T.B.L.; Software: C.B., J.C.-D; Formal analysis: T.B.L., C.B.; Data curation: T.B.L.; Writing - original draft: T.B.L.; Writing - review & editing: T.B.L., C.B., J.C.-D; Supervision: J.C.-D.

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References
Baugh, A. T., Bee, M. A. and Gall, M. D. (2019). The paradox of hearing at the lek: auditory sensitivity increases after breeding in female gray treefrogs (*Hyla chrysoscelis*). *J. Comp. Physiol. A* **205**, 629-639. doi:10.1007/s00359-019-01354-0

Berlin, C. I., Hood, L. J., Barlow, E. K., Morehouse, C. R. and Smith, E. G. (1991). Derived guinea pig compound VIIIth nerve action potentials to continuous pure tones. *Hear. Res.* **52**, 271-280. doi:10.1016/0378-5955(91)90017-4

Brandt, C., Andersen, T. and Christensen-Dalsgaard, J. (2007). Demonstration of a portable system for auditory brainstem recordings based on pure tone masking difference. In *Auditory signal processing in hearing-impaired listeners. 1st International Symposium on auditory and Audiological Research* (ed. T. Dau, J. M. Buchholz, J. M. Harte and T. U. Christiansen), pp. 241-247. Copenhagen: Centertryk.

Brandt, C., Brande-Lavridsen, N. and Christensen-Dalsgaard, J. (2018). The Masked ABR (mABR): a new measurement method for the auditory brainstem response. *J. Assoc. Res. Otolaryngol.* **19**, 753-761. doi:10.1007/s10162-018-00696-x

Brittan-Powell, E. F., Dooling, R. J. and Gleich, O. (2002). Auditory brainstem responses in adult budgerigars (*Melopsittacus undulatus*). *J. Acoust. Soc. Am.* **112**, 999-1008. doi:10.1121/1.1494807

Brittan-Powell, E. F., Christensen-Dalsgaard, J., Tang, Y., Carr, C. and Dooling, R. J. (2010). The auditory brainstem response in two lizard species. *J. Acoust. Soc. Am.* **128**, 787-794. doi:10.1121/1.3458813

Brzoska, J., Walkowiak, W. and Schneider, H. (1977). Acoustic communication in the grass frog (*Rana t. temporaria L.*): calls, auditory thresholds and behavioral responses. *J. Comp. Physiol.* **118**, 173-186. doi:10.1007/BF00611821

Buerkle, N. P., Schrode, K. M. and Bee, M. A. (2014). Assessing stimulus and subject influences on auditory evoked potentials and their relation to peripheral physiology in green treefrogs (*Hyla cinerea*). *Comp. Biochem. Physiol A Mol. Integr. Physiol.* **178**, 68-81. doi:10.1016/j.cbpa.2014.08.005

Burkard, R. F. (2007). The auditory brainstem response. In *Auditory Evoked Potentials: Basic Principles and Clinical Application* (ed. R. F. Burkard, J. J. Eggermont and M. Don), pp. 229-253. Lippincott Williams & Wilkins.

Christensen-Dalsgaard, J., Jørgensen, M. B. and Kanneworff, M. (1998). Basic response characteristics of auditory nerve fibers in the grassfrog (*Rana temporaria*). *Hear. Res.* **119**, 155-163. doi:10.1016/S0378-5955(98)00047-1

Christensen-Dalsgaard, J., Brandt, C., Willis, K. L., Christensen, C. B., Ketten, D., Edds-Walton, P., Fay, R. R., Madsen, P. T. and Carr, C. E. (2012). Specialization for underwater hearing by the tympanic middle ear of the turtle, *Trachemys scripta elegans*. *Proc. R. Soc. B Biol. Sci.* **279**, 2816-2824. doi:10.1098/rspb.2012.0290

Christensen, C. B., Lauridsen, H., Christensen-Dalsgaard, J., Pedersen, M. and Madsen, P. T. (2015a). Better than fish on land? Hearing across metamorphosis in salamanders. *Proc. R. Soc. B Biol. Sci.* **282**, 20141943. doi:10.1098/rspb.2014.1943

Christensen, C. B., Christensen-Dalsgaard, J. and Madsen, P. T. (2015b). Hearing of the African lungfish (*Protopterus annectens*) suggests underwater pressure detection and rudimentary aerial hearing in early tetrapods. *J. Exp. Biol.* **218**, 381-387. doi:10.1242/jeb.116012

Corwin, J. T., Bullock, T. H. and Schweitzer, J. (1982). The auditory brain stem response in five vertebrate classes. *Electroencephalogr. Clin. Neurophysiol.* **54**, 629-641. doi:10.1016/0013-4694(82)90117-1

Eatock, R. A. and Manley, G. A. (1981). Auditory nerve fibre activity in the Tokay gecko - II. Temperature effect on tuning. *J. Comp. Physiol.* **142**, 219-226. doi:10.1007/BF00605740

Egner, S. A. and Mann, D. A. (2005). Auditory sensitivity of sergeant major damselfish *Abudefduf saxatilis* from post-settlement juvenile to adult. *Mar. Ecol. Prog. Ser.* **285**, 213-222. doi:10.3354/meps285213

Elepfandt, A., Eistetter, I., Fleig, A., Günther, E., Hainich, M., Hepperle, S. and Traub, B. (2000). Hearing threshold and frequency discrimination in the purely aquatic frog *Xenopus laevis* (Pipidae): measurement by means of conditioning. *J. Exp. Biol.* **203**, 3621-3629.

Fay, R. R. (1978). Phase-locking in goldfish saccular nerve fibres accounts for frequency discrimination capacities. *Nature* **275**, 320-322. doi:10.1038/275320a0

Goutte, S., Mason, M. J., Christensen-Dalsgaard, J., Montealegre-Z, F., Chivers, B. D., Sarria-S, F. A., Antoniazzi, M. M., Jared, C., Almeida Sato, L. and Felipe Toledo, L. (2017). Evidence of auditory insensitivity to vocalization frequencies in two frogs. *Sci. Rep.* **7**, 12121. doi:10.1038/s41598-017-12145-5

Kenyon, T. N., Ladich, F. and Yan, H. Y. (1998). A comparative study of hearing ability in fishes: the auditory brainstem response approach. *J. Comp. Physiol. A* **182**, 307-318. doi:10.1007/s003590050181

Kraus, N. and Nicol, T. (2009). Auditory evoked potentials. In *Encyclopedia of Neuroscience* (ed. M. D. Binder, N. Hirokawa and U. Windhorst), pp. 214-218. Springer Berlin Heidelberg.

Ladich, F. and Fay, R. R. (2013). Auditory evoked potential audiometry in fish. *Rev. Fish Biol. Fish.* **23**, 317-364. doi:10.1007/s11160-012-9297-z

Martin, K. J., Alessi, S. C., Gaspard, J. C., Tucker, A. D., Bauer, G. B. and Mann, D. A. (2012). Underwater hearing in the loggerhead turtle (*Caretta caretta*): a comparison of behavioral and auditory evoked potential audiograms. *J. Exp. Biol.* **215**, 3001-3009. doi:10.1242/jeb.066324

Maxwell, A., Hansen, K. A., Larsen, O. N., Christensen-Dalsgaard, J., Wahlberg, M. and Siebert, U. (2016). Testing auditory sensitivity in the great cormorant (Phalacrocorax carbo sinensis): Psychophysics vs. auditory brainstem response. *Proc. Meet. Acoust.* **27**, 1-8. doi:10.1121/2.0000261

Mooney, T. A., Hanlon, R. T., Christensen-Dalsgaard, J., Madsen, P. T., Ketten, D. R. and Nachtigall, P. E. (2010). Sound detection by the longfin squid (*Loligo pealeii*) studied with auditory evoked potentials: sensitivity to low-frequency particle motion and not pressure. *J. Exp. Biol.* **213**, 3748-3759. doi:10.1242/jeb.048348

Narins, P. M. (1987). Coding of signals in noise by amphibian auditory nerve fibers. *Hear. Res.* **26**, 145-154. doi:10.1016/0378-5955(87)90106-7

Narins, P. M. and Hillery, C. M. (1983). *Frequency Coding in the Inner Ear of Anuran Amphibians.p*. Berlin: Springer Berlin Heidelberg.

Piniak, W. E. D., Mann, D. A., Harms, C. A., Jones, T. T. and Eckert, S. A. (2016). Hearing in the juvenile green sea turtle (Chelonia mydas): a comparison of underwater and aerial hearing using auditory evoked potentials. *PLoS ONE* **11**, e0159711. doi:10.1371/journal.pone.0159711

Schmitz, B., White, T. D. and Narins, P. M. (1992). Directionality of phase locking in auditory nerve fibers of the leopard frog *Rana pipiens pipiens*. *J. Comp. Physiol. A* **170**, 589-604. doi:10.1007/BF00199335

Schrode, K. M., Buerkle, N. P., Brittan-Powell, E. F. and Bee, M. A. (2014). Auditory brainstem responses in Cope's gray treefrog (*Hyla chrysoscelis*): effects of frequency, level, sex and size. *J. Comp. Physiol. A* **200**, 221-238. doi:10.1007/s00359-014-0880-8

Shapiro, S. S. and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591-611. doi:10.1093/biomet/52.3-4.591

Taylor, R. C., Akre, K., Wilczynski, W. and Ryan, M. J. (2019). Behavioral and neural auditory thresholds in a frog. *Curr. Zool.* **65**, 333-341. doi:10.1093/cz/zoy089

Vidler, M. and Parker, D. (2004). Auditory brainstem response threshold estimation: subjective threshold estimation by experienced clinicians in a computer simulation of the clinical test. *Int. J. Audiol.* **43**, 417-429. doi:10.1080/14992020400050053

Womack, M. C., Christensen-Dalsgaard, J., Coloma, L. A., Chaparro, J. C. and Hoke, K. L. (2017). Earless toads sense low frequencies but miss the high notes. *Proc. R. Soc. B Biol. Sci.* **284**, 20171670. doi:10.1098/rspb.2017.1670

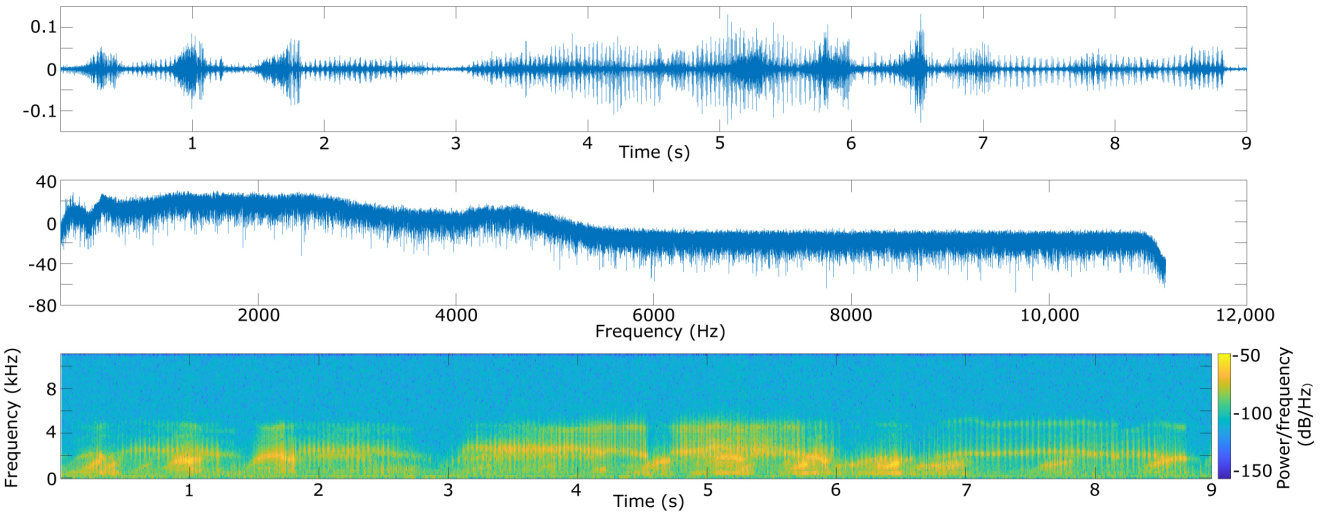

Fig. S1: **Oscillogram, frequency spectrum, and spectrogram of a field recording of calling Green frogs (P. esculentus).** Spectrogram settings: FFT size 128, overlap 98%, 128 points Hamming window.

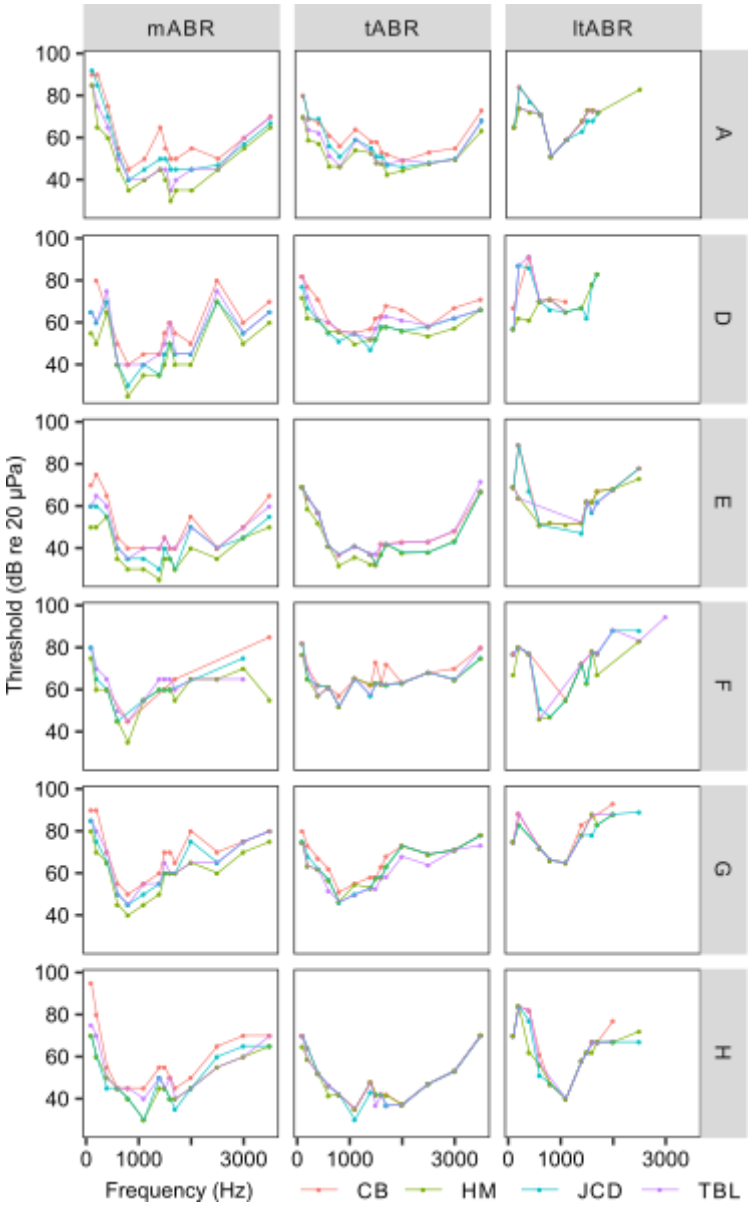

Fig. S2: **Subjectively determined audiograms for the three ABR methods in individuals A and D to H.** Colors represent different researchers.