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The representation of conspecific sounds in the auditory brainstem of teleost fishes

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Summary

Temporal patterns of sounds are thought to be the most important carriers of acoustic information in teleost fishes. In order to investigate how conspecific sounds are processed by the auditory system, auditory brainstem responses (ABRs) elicited by conspecific sounds were recorded in five species of teleosts. In the catfishes *Platydoras costatus* and *Pimelodus pictus*, the loach *Botia modesta* and the labyrinth fish *Trichopsis vittata*, all of which are hearing specialists, each pulse within the sounds elicited a separate brainwave that closely followed the temporal structure. The ABRs of *P. costatus* and *B. modesta* also represent amplitude patterns of conspecific sounds. By contrast, ABRs of the sunfish *Lepomis gibbosus*, a hearing non-specialist, consisted of long series of waves that could not be attributed to specific sound pulses. A more detailed analysis, however, indicated that each stimulus pulse contributed to the compound ABR waveform. Spectral analysis of low-pitched drumming sounds of *P. pictus* and corresponding ABRs showed peaks in the ABR spectra at the harmonics of the sound. Our results indicate that, besides temporal patterns, amplitude fluctuations and the frequency content of sounds can be represented in the auditory system and help the fish to extract important information for acoustic communication.

Key words: auditory brainstem response, conspecific sound, temporal sound pattern, teleost, acoustic information, *Platydoras costatus*, *Pimelodus pictus*, *Botia modesta*, *Trichopsis vittata*, *Lepomis gibbosus*.

Introduction

Bony fishes have evolved a variety of sound-producing mechanisms and acoustic signals. These can play an important role in intraspecific communication, mainly during mating (e.g. in damselfishes, Myrberg et al., 1986; sunfishes, Gerald, 1971; toadfishes, McKibben and Bass, 1998; mormyrids, Crawford, 1997a; and catfish, Pruzsinszky and Ladich, 1998) and during agonistic interactions (for a review, see Ladich, 1997a). Temporal characteristics of sounds are thought to be important carriers of information in teleost fishes because they frequently consist of series of short broadband pulses (e.g. stridulatory sounds of gouramis and catfishes; Ladich et al., 1992; Ladich, 1997b). Moreover, distinct temporal patterns often distinguish sounds of closely related species, such as in sunfishes (Gerald, 1971), pomacentrids (Spanier, 1979), mormyrids (Marvit and Crawford, 2000), gadoids (Hawkins and Rasmussen, 1978) and gouramis (Ladich et al., 1992). Behavioral experiments have shown that fishes can respond selectively to sounds that differ in their temporal patterns. Winn (1964), for example, demonstrated that the oyster toadfish *Opsanus tau* can selectively alter its own calling rate when exposed to playbacks of boat-whistle sounds at different rates. This discriminatory ability led the author to conclude that temporal coding is important for the communication system in toadfishes. Based on behavioral discrimination tasks, damselfishes of the genus *Pomacentrus* can selectively distinguish conspecific sounds from those of closely related congenerics based on temporal patterns, especially pulse periods (Spanier, 1979). During playback experiments in the field, Gerald (1971) reported that some sunfishes of the genus *Lepomis* can recognize conspecific grunts based on their temporal structure.

Temporal sound patterns are also important in the communication system of other animal groups such as frogs and insects. In the Pacific tree frog *Hyla regilla*, two different call types that elicit distinct behavior are discriminated based on interpulse intervals (Rose and Brenowitz, 2002). In many acoustically active insects, information about species identity is primarily encoded in the temporal structure of the song (for a review, see Römer, 1998).

To date, only few data are available showing that the auditory system of fishes is capable of resolving acoustic differences based on temporal patterns. Marvit and Crawford (2000) showed that weakly electric fish of the genus *Pollimyrus* can distinguish interclick intervals (ICIs) below 1 ms; moreover, their tone frequency and click-rate detection thresholds indicate that natural sounds of *Pollimyrus* could mediate species and individual recognition. Analyzing the auditory brainstem response (ABRs) to double-click stimuli with varying click periods, Wysocki and Ladich (2002)

showed that the minimum click period resolvable by the auditory system was below 1.5 ms in five hearing specialists. But how is a complex species-specific sound consisting of several pulses varying in pulse periods and amplitudes represented in the auditory system? Does this high resolution capability and reliability of representation in the auditory pathway also hold true for a series of repeated pulses, or do habituation or inhibition processes take place?

In order to answer these questions, we recorded ABRs evoked by conspecific sounds in different species. Bullock and Ridgway (1972), using alert porpoises (*Tursiops truncatus*), proved that ABRs to conspecific sounds can be obtained. The first aim was to determine if and how complex conspecific sounds are represented by the auditory system in fishes possessing different sound-producing mechanisms and hearing abilities. In a second step, we investigated which acoustical variables in communication sounds – time, frequency and/or amplitude – are encoded in the auditory brainstem. Finally, for the first time, we directly investigated the auditory sensitivity to conspecific sounds. We investigated four representative hearing specialists, which possess accessory hearing structures that enhance hearing sensitivity and the frequency range perceived. In addition, we tested a hearing generalist lacking accessory hearing structures, whose hearing range is limited to the detection of lower frequency sounds (<1 kHz) of higher intensities. Within the specialists, we investigated evoked responses of the lined Raphael catfish *Platydoras costatus* (Doradidae) and *Pimelodus pictus* (Pimelodidae) to conspecific broadband stridulatory sounds and of *P. pictus* also to the low-frequency drumming sounds. Furthermore, we tested orangefin loaches *Botia modesta* (Cobitidae), which produce high-intensity, broadband knocking sounds emitted singly or in series (Ladich, 1999), and croaking gouramis *Trichopsis vittata* (Belontiidae), which produce broadband double-pulsed sounds (Kratochvil, 1985). Among hearing generalists, we chose the pumpkinseed sunfish *Lepomis gibbosus*, which produces broadband, rasping sounds with variable pulse patterns and pulse durations (Ballantyne and Colgan, 1978).

Materials and methods

Animals

Test subjects were obtained from local pet suppliers, except for *T. vittata*, which were laboratory reared. All animals were kept in planted aquaria with sand at the bottom, equipped with half flower pots as hiding places, filtered by external filters and maintained at a 12 h:12 h L:D cycle. The fish were fed live *Tubifex* sp., chironomid larvae or commercially prepared flake food (Tetramin®; TetraGmbH, Melle, Germany) daily. Efforts were made to provide a quiet environment (e.g. no submerged filters or air stones). Test subjects were lined Raphael catfish *Platydoras costatus* (Linnaeus 1766) (*N*=7; 97–110 mm standard length; 21.2–29.3 g body mass), *Pimelodus pictus* Steindachner 1876 (*N*=7; 63–86 mm; 4.3–10.8 g), pumpkinseed sunfish *Lepomis gibbosus* (Linnaeus 1758) (*N*=6; 59–78 mm; 6.1–16.8 g), croaking gouramis *Trichopsis vittata* (Cuvier and Valenciennes 1831) (*N*=6; 36–43 mm; 0.9–2.2 g) and orangefin loaches *Botia modesta* Bleeker 1864 (*N*=6; 51–69 mm; 3.3–8.0 g). All experiments were performed with the permission of the Austrian Commission on Experiments in Animals (GZ 68.210/30-Pr/4/2001).

Auditory brainstem response recordings

The ABR recording protocol used in this study followed that recently described in Wysocki and Ladich (2001, 2002). Therefore, only a brief summary of the basic technique is given here. During the experiments, the fish were mildly immobilized with Flaxedil (gallamine triethiodide; Sigma Aldrich Handels GmbH, Vienna, Austria). The dosage used was 1.3–1.6 μg g^{-1} for *P. costatus*, 2.7–5.9 μg g^{-1} for *P. pictus*, 1.9–4.9 μg g^{-1} for *L. gibbosus*, 0.3–0.5 μg g^{-1} for *T. vittata* and 3.3–7.5 μg g^{-1} for *B. modesta*. This dosage allowed the fish to retain slight opercular movements during the experiments but without significant myogenic noise to interfere with the recording. Test subjects were secured in a plastic bowl (37 cm diameter, 8 cm water depth, 2 cm layer of fine sand) lined on the inside with acoustically absorbent material (closed cell foam); in a previous study (Wysocki and Ladich 2002), this proved to reduce resonances and reflections and thus to preserve the temporal structure of broadband stimuli. Fish were positioned under water such that the skin region between the nares and the medulla was 1 mm above the surface; thus, the contacting points between skin and electrodes were not in the water. A respiration pipette was inserted into the subject's mouth. Respiration was achieved through a simple temperature-controlled (24±1°C), gravity-fed water circulation system. The ABRs were recorded using silver wire electrodes (0.25 mm diameter) pressed firmly against the skin. The portion of the head above the water surface was covered by a small piece of Kimwipes tissue paper to keep it moist and to ensure proper contact during experiments. The recording electrode was placed in the midline of the skull over the region of the medulla, and the reference electrode was placed cranially between the nares. Shielded electrode leads were attached to the differential input of an AC preamplifier (Grass P-55, gain 100×, high-pass at 30 Hz, low-pass at 1 kHz). The plastic tub was positioned on an air table (TMC Micro-g 63-540; Technical Manufacturing Corporation, Peabody, MA, USA) that rested on a vibration-isolated concrete plate. The entire set-up was enclosed in a walk-in soundproof room, which was constructed as a Faraday cage (interior dimensions: 3.2 m×3.2 m×2.4 m).

Sound stimuli were presented and ABR waveform recorded using a modular rack-mount system [Tucker-Davis Technologies (TDT), Gainesville, FL, USA] controlled by an optically linked Pentium PC containing a TDT digital-processing board and running TDT BioSig 3.2 software.

Sounds presented

Sound stimuli for hearing specialists were chosen among previously recorded (Ladich, 1998, 1999; Wysocki and Ladich, 2001, 2002) representative conspecific sounds. All stimuli

were complete sounds as emitted by the fish, except the drumming sound of *P. pictus*, which was shortened by approximately half. For *T. vittata*, a sound consisting of three pairs of pulses was taken in order to avoid a measuring time that was too long. Among *B. modesta*, which emit single or a series of knocks with a long pulse period, we chose one sound consisting of two and another of three knocks. For the hearing generalist *L. gibbosus*, a sound consisting of four pulses was chosen from field recordings provided by Kurt Osterwald. A second sound stimulus was created by eliminating the second and third pulses of the four-pulsed test stimulus using CoolEdit 2000 (Syntrillium Software Corporation, Phoenix, AZ, USA).

In addition, control tests using heterospecific sounds were performed in order to test whether the responses observed are specific to conspecific sounds. Sounds of *L. gibbosus*, *T. vittata* and *B. modesta* (three-pulsed sound) were presented to four individuals of *P. pictus*, and sounds of *T. vittata*, *B. modesta* (three-pulsed sound) and *P. pictus* (stridulation sound) were presented to four individuals of *L. gibbosus*.

All sound wave files were imported into TDT SigGen 3.2 software and fed through a DA1 digital–analog converter, a PA4 programmable attenuator and a power amplifier (Denon PMA 715R, Alesis RA300). A dual-cone speaker (Tannoy System 600, frequency response 50 Hz–15 kHz±3 dB), mounted 1 m in the air above test subjects, was used to present the stimuli during testing. Stimuli were presented to the animals at repetition rates of 2–10 per second according to the length of the stimulus. A hydrophone (Brüel & Kjaer 8101; Nürum, Denmark; frequency range, 1 Hz–80 kHz±2 dB; voltage sensitivity, –184 dB re 1 V μPa^{-1}) was placed close to the right side of the animals (2 cm away) in order to control for stimulus characteristics [such as sound pressure level (SPL), sound spectrum and temporal structure]. SPLs of sound stimuli were measured by a Brüel & Kjaer 2238 Mediator, Brüel & Kjaer 2804 power supply and Brüel & Kjaer hydrophone 8101 (time weighting, RMS Fast; frequency weighting, linear between 20 Hz and 20 kHz). For each test condition, stimuli were presented at opposite polarities and the ABRs to the two stimulus phases were averaged by the BioSig software in order to eliminate stimulus artefacts. In order to create a 180° phase-shifted stimulus, a copy of each original signal was inverted by 180° using CoolEdit 2000 (for illustration, see Fig. 1). Each response waveform represents an average of 1400–2000 stimulus presentations over an analysis window of 50–400 ms using a sampling rate of 20 kHz. Sound pressure levels of stimuli were reduced in 4 dB steps until the ABR waveform disappeared. The lowest SPL for which a repeatable ABR trace to any of the presented sound pulses could be obtained, as determined by overlaying replicate traces, was considered the threshold (Kenyon et al., 1998).

ABR waveform analysis and statistics

The following characteristics of sounds and brainstem responses were analyzed: number, latencies, amplitude and frequency content of responses.

Latencies of the response were defined as the time interval between the onset of the sound stimulus and the first negative peak of the ABR waveform. Amplitudes were measured from the first negative peak to the most constant positive peak in each species. Wherever a given waveform could be related to a corresponding pulse of the sound stimulus, latencies and amplitudes of the ABRs and the sound pulses 20 dB above the mean hearing threshold of each particular species to this sound were compared by two-tailed correlations [Pearson's correlation coefficient was used when a previous Kolmogorov–Smirnov test showed that data/sound stimulus characteristics (temporal structure, peak amplitudes) were normally distributed]. All statistical tests were run using SPSS version 10.0.

In order to analyze whether the main frequency content of sounds was represented within ABRs, fast Fourier transformations (FFTs) of sound stimulus waveforms and corresponding ABR waveforms were performed using S_Tools, the Integrated Workstation for Acoustics, Speech and Signal Processing developed by the Research Laboratory of Acoustics at the Austrian Academy of Sciences. Only sound stimuli with a main energy content below 1 kHz were analyzed because of the filter settings of the electrode preamplifier (low-pass at 1 kHz) and because human ABR audiometry has revealed that there is little spectral ABR energy at frequencies above 2 kHz. Evoked-potential studies on mammals showed that certain frequency contents of sounds, such as formants of vowels (Krishnan, 2002), are reflected by scalp recorded auditory potentials; this is attributed to phase-locked activity in populations of neural elements within the brainstem. Spectral peaks were compared between the FFT spectrum of the stimulus and the corresponding mean ABR spectrum generated using individual spectral data of each species. Spectral analyses included the whole drumming sound stimulus of *P. pictus*, the first pulse of the two-pulsed sound and the third pulse of the three-pulsed sound of *B. modesta* and their corresponding ABRs. Only a single pulse of each sound of *B. modesta* was chosen, because the intervals between pulses were so long that an averaged spectrum would have contained a large amount of background noise, possibly influencing the spectrum. For the same reason, spectral analysis in *L. gibbosus* concentrated on the first pulse of the sound in which pulses 2 and 3 were omitted; this was also the only pulse to which a separate ABR trace could be attributed and it would have been difficult to interpret a spectrum of a waveform consisting of several superimposed ABR traces.

Results

Representation of conspecific sounds

Representation of temporal patterns within ABRs

Presenting sound stimuli of opposite polarities efficiently eliminated potential stimulus artefacts in ABR traces because the ABR traces were unaffected by changes in polarity of the stimulus when averaged (Fig. 1). In contrast to ABR waveforms, traces of sounds of opposite polarities always

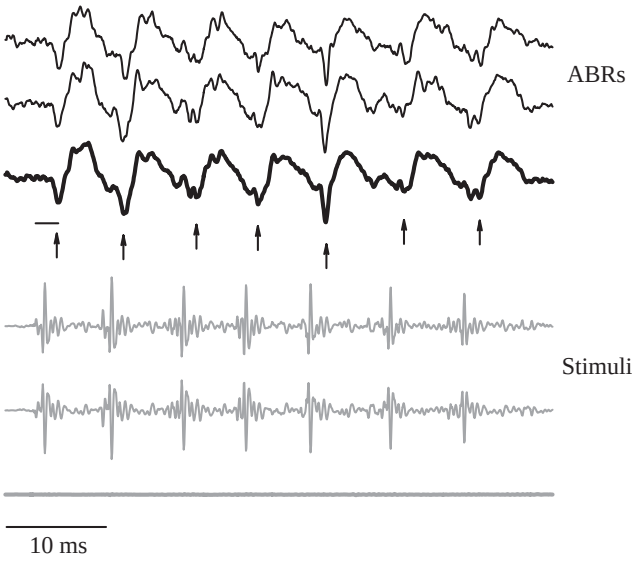

Fig. 1. Auditory brainstem response (ABR) waveforms (dark lines) of *P. pictus* in response to stridulation-sound stimuli (light traces) presented at opposite polarities and the corresponding stimuli. Bold traces are the mean of each pair. The arrows indicate the reference peaks for the measurements of latencies. The small horizontal bar at the left side indicates the latency of the first ABR wave.

cancelled each other out when averaged. Fig. 1 illustrates this *via* the responses to pectoral stridulation sounds of the catfish *P. pictus*. Each pulse of each conspecific sound elicited a separate ABR waveform in all four hearing specialists (Fig. 2). The onsets of the single pulses within a sound and the onsets of the first negative pulse of each corresponding ABR wave were highly correlated (r=1, P<0.001) for each hearing specialist and sound type. This indicates that the temporal structure of conspecific sounds is exactly represented in the auditory brainstem of the fishes. For *P. costatus*, the sound stimulus consisted of a series of 11 pulses with varying amplitude and pulse periods ranging from 6.6 ms to 10.2 ms. Detailed analysis of the ABR wave latencies (defined as onset of the first negative ABR wave minus stimulus onset) showed a variance of mean latencies to each pulse (Table 1). When ABR latency values were correlated to the amplitude of the corresponding stimulus pulse, a significant negative correlation was observed (r=–0.858, P<0.001). This means that a higher stimulus pulse amplitude evoked an auditory response with a shorter latency than a less-intensive pulse.

The stridulation sound stimulus for *P. pictus* consisted of seven consecutive pulses of variable amplitude with pulse periods from 5.9 ms to 7.6 ms (Fig. 2A). Latencies of the corresponding ABR waves varied (Table 1) but showed a significant negative correlation to stimulus pulse amplitudes (r=–0.778, P<0.05), similar to *P. costatus*.

In *B. modesta*, the latencies to the two tested stimuli differed from one another considerably: the three short pulses (pulse periods 145.9 ms and 170.9 ms) evoked a fast response, whereas the sounds consisting of two longer pulses (pulse period 125.4 ms) caused a longer latency in ABR waveforms (Fig. 2B; Table 1).

The sound stimulus for *T. vittata* consisted of three pairs of pulses (Fig. 2C), each pair representing the alternating plucking of two enhanced tendons over the bony basis of the rays of two pectoral fins. Single-pulse periods within a pair of pulses were 6.3 ms, 10.9 ms and 6.9 ms and double-pulse periods were 47.5 ms and 45 ms. ABR waves to the separate stimulus pulses of croaking gouramis had the shortest overall latencies of all species (Table 1). Similar to both catfishes, ABR latencies were also negatively correlated to stimulus pulse amplitudes (r=–0.828, P<0.05).

In contrast to hearing specialists, specific ABR waves in *L. gibbosus* could not be attributed to the separate pulses of the complete conspecific sound stimulus (Fig. 2C) consisting of

Table 1. *Latencies (mean ± S.E.M.) of the first negative peak of auditory brainstem response (ABR) waves to the separate pulses within a species-specific sound stimulus*

Pulse number	*Pc* (N=7)	*Pp* (N=7)	*Bm2* (N=6)	*Bm3* (N=6)	*Tv* (N=7)	*Lg* (N=6)
1	2.41±0.17	1.81±0.04	4.55±0.02	1.73±0.02	1.36±0.24	1.78±0.32
2	2.22±0.15	1.72±0.06	4.73±0.01	2.07±0.03	1.23±0.22	2.68±0.60
3	2.34±0.15	1.75±0.05	–	1.63±0.12	1.23±0.23	–
4	2.17±0.12	1.69±0.06	–	–	1.22±0.21	3.28±0.47
5	2.12±0.11	1.93±0.05	–	–	1.20±0.23	–
6	2.11±0.13	1.83±0.07	–	–	1.25±0.21	–
7	2.05±0.12	1.99±0.05	–	–	–	–
8	2.01±0.14	–	–	–	–	–
9	2.16±0.18	–	–	–	–	–
10	2.13±0.11	–	–	–	–	–
11	2.41±0.14	–	–	–	–	–

All sounds were played back at 20 dB (RMS fast) above mean hearing threshold of each species. *Pc*, *Platydoras costatus*; *Pp*, *Pimelodus pictus*; *Bm2*, *Botia modesta* two-pulsed sound; *Bm3*, *B. modesta* three-pulsed sound; *Tv*, *Trichopsis vittata*; *Lg*, *Lepomis gibbosus* (latency values of pulses 1 and 4 from the modified sound with pulses 2 and 3 omitted; values for pulse 2 were measured after a point-to-point subtraction of the response to the complete conspecific sound from the modified sound; see also Fig. 3).

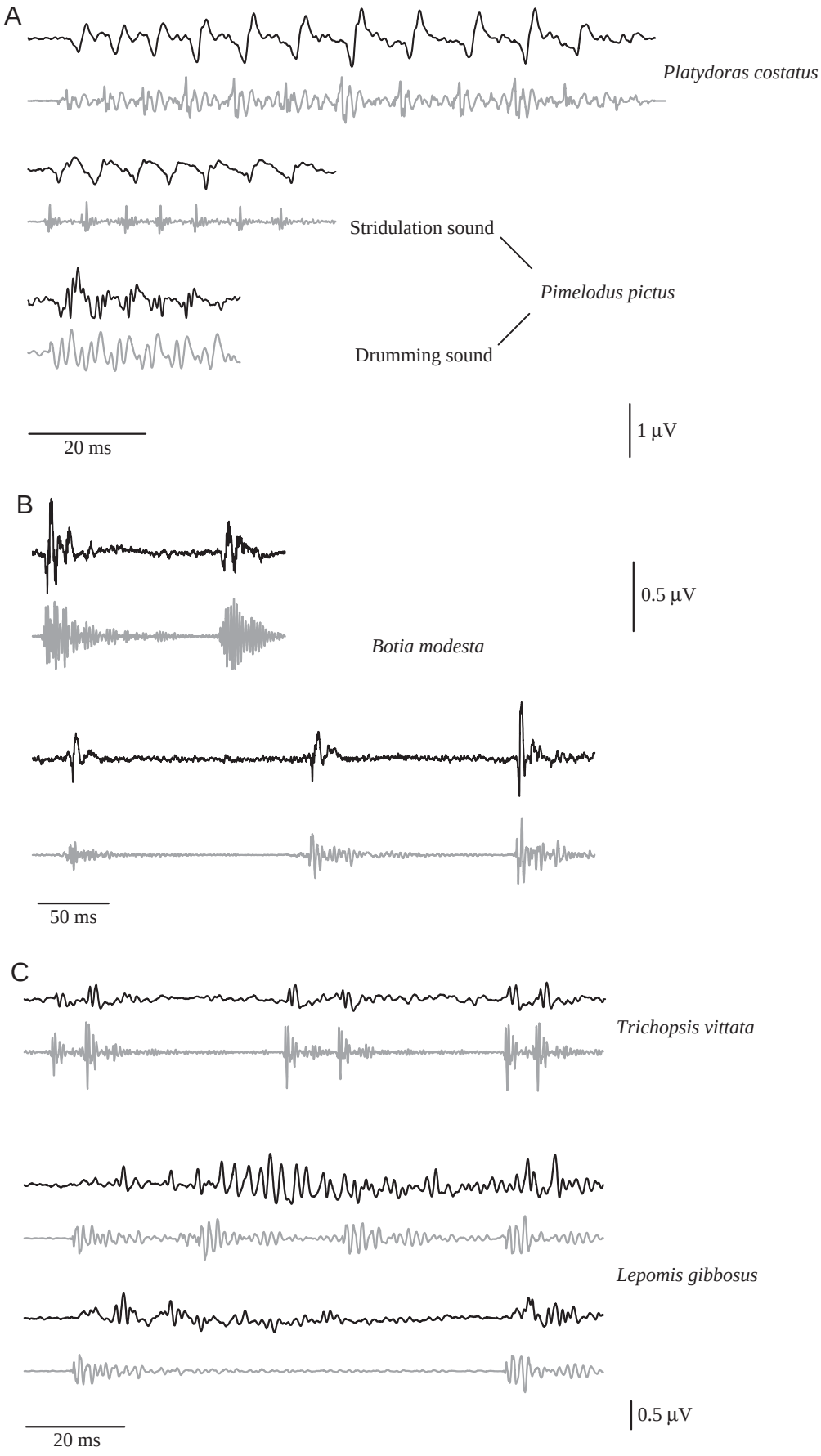

Fig. 2. Auditory brainstem response (ABR) waveforms (dark traces) and oscillograms of each sound stimulus (light traces) of the different species investigated at 20 dB above mean hearing threshold of each particular species within (A) siluriforms, (B) cypriniforms and (C) perciforms. All stimuli were recorded under water with the hydrophone 2 cm away from the animals. The amplitudes of the sound waveforms were adjusted to fit to the proportions of the ABR waveforms.

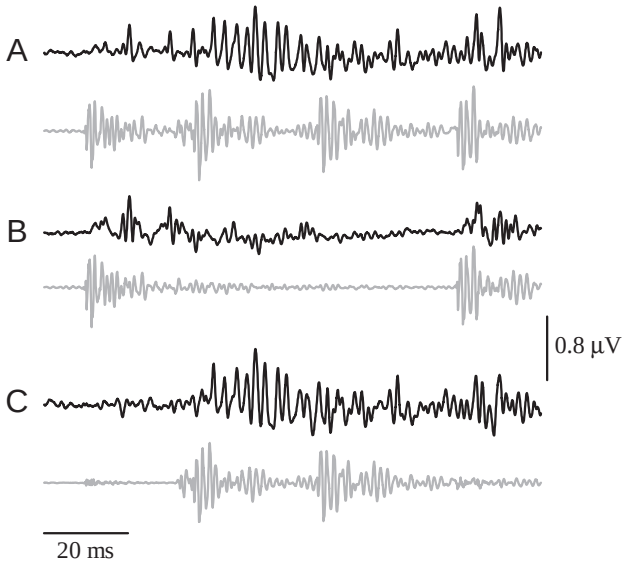

Fig. 3. Auditory brainstem response (ABR) waveforms (dark traces) and oscillograms of stimuli (light traces) of (A) the natural sound, (B) the modified sound with pulses 2 and 3 omitted and (C) the resulting waveforms after a point-to-point subtraction of B from A of *L. gibbosus*. For further details, see Fig. 2.

four pulses (pulse periods 25.6 ms, 29.4 ms and 33.3 ms). When pulses 2 and 3 were omitted from the sound stimulus, a response to the first pulse lasting approximately 51.7 ms could be differentiated from the response to the second pulse 88.3 ms apart (Fig. 3). The response to the second pulse was also longer than those of the hearing specialists, but it exceeded the duration of our 120 ms time window and was therefore not measurable. The first discernible ABR deflection started about 1.8 ms after onset of the first stimulus pulse of the sounds. For the modified sound (pulses 2 and 3 omitted, pulse period 88.3 ms), the mean latency to pulse 4 was 3.2 ms (Table 1). The lack of four separate responses to the unmodified sounds does not necessarily mean that pulses 2 and 3 are not represented in the auditory brainstem of the fish: the responses are quite long and could simply be superimposed. In order to test this for at least pulse 2, a point-to-point subtraction procedure (for details see Wysocki and Ladich, 2002) was performed: the response to the modified sound was subtracted from the response to the complete sound. After this subtraction, a response to pulse 2 was discernible with a mean latency intermediate between that of pulses 1 and 4 (Fig. 3; Table 1), to which a response to pulse 3 could be superimposed.

Representation of amplitude patterns within ABRs

Correlating the amplitudes of conspecific sound pulses to the amplitude of the corresponding ABR waves revealed differences between species: a significant correlation in amplitude was measured in *P. costatus* (r=0.570, P<0.001; Fig. 4A) and in *B. modesta* for both sound stimuli (r=0.705, P<0.001 for the sound consisting of two longer pulses, and r=0.799, P<0.001 for the sound consisting of three short

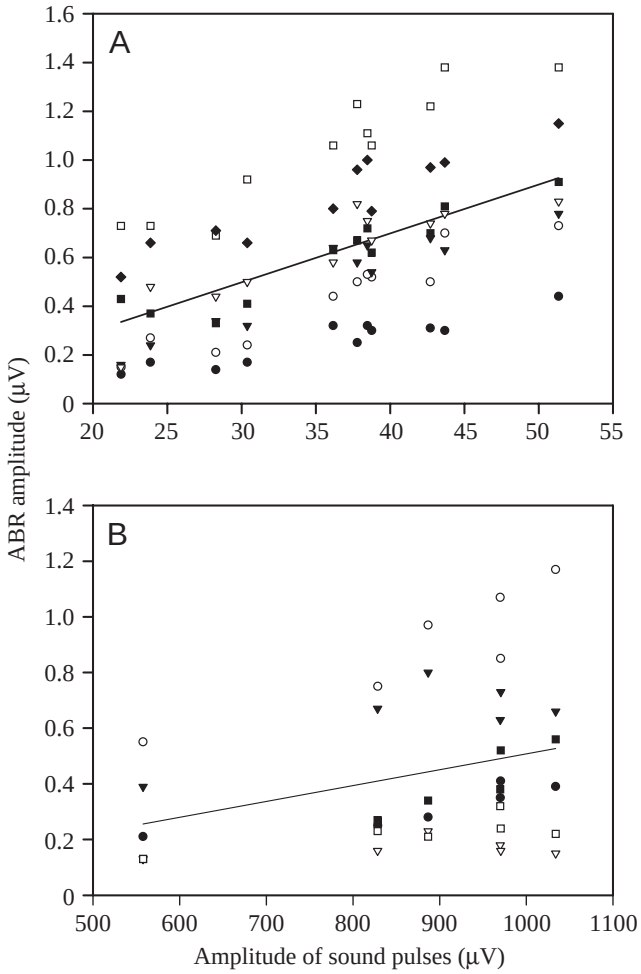

Fig. 4. Correlation between stimulus pulse amplitudes and amplitudes of the corresponding auditory brainstem response (ABR) waveforms in (A) *P. costatus* and (B) *T. vittata*. The different symbols represent different individuals tested. Regression equations are: ABR amplitude=amplitude of sound pulses×(0.02–0.103) for A, and ABR amplitude=amplitude of sound pulses×(0.00057–0.062) for B.

pulses). In *T. vittata*, the correlation was close to significance (r=0.314, P=0.062; Fig. 4B). In *P. pictus*, neither amplitudes of the stridulation sound (r=0.251, P=0.082) nor of the drumming sound (r=–0.146, P=0.368) were correlated significantly to the amplitudes of the corresponding ABR waves. Because of the lack of clear, short, separated ABRs to each stimulus pulse, no such correlation could be made in *L. gibbosus*.

When approaching the hearing thresholds, brainwaves evoked by the less-intensive stimulus pulses disappeared: at threshold, an individually different number of the more-intensive sound pulses – usually at mid-stridulation – elicited a response in the two catfishes. For the drumming sound of *P. pictus*, the response to the first pulse always persisted at hearing threshold, while a variable number of the subsequent pulses disappeared in the different individuals. In *T. vittata*, the first pulse of the first double pulse, which had a considerably

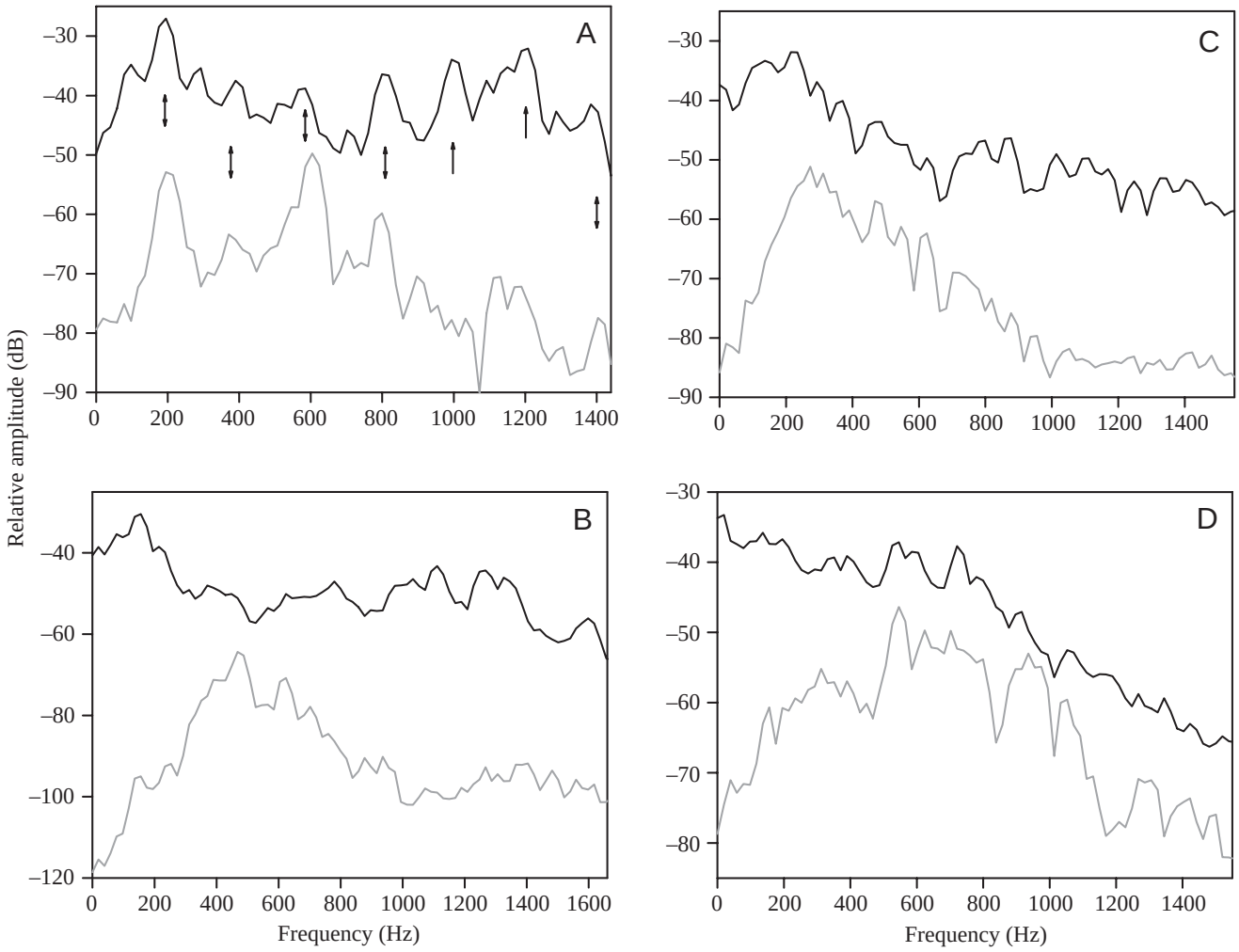

Fig. 5. Fast Fourier transformations (FFTs) of sound stimuli recorded by the hydrophone 2 cm away from the fish (light traces) and the auditory brainstem response (ABR) waves elicited (dark traces). (A) The drumming sound of *P. pictus*, (B) the two-pulsed sound of *B. modesta*, (C) the three-pulsed sound of *B. modesta* and (D) the sound of *L. gibbosus*. All stimuli and ABRs were recorded at 25 dB above mean hearing threshold of each particular species (30 dB for C). The double-headed arrows in A indicate harmonics of the stimulus that correspond to spectral peaks of the ABRs; the single arrows indicate spectral peaks of the ABR waveforms at frequencies representing additional harmonics of the stimulus. Note the different frequency scalings for each species. The error of the frequency axis is ±20 Hz due to the bandwidth settings of the FFT calculations.

lower amplitude than the rest of the stimulus (Fig. 2C), evoked no ABR at hearing threshold. In *B. modesta*, at hearing threshold, the pulse with the highest amplitude (pulse 1 of the low-frequency sound, pulse 3 of the broadband sound) was always last represented in the ABR. In *L. gibbosus*, it was not possible to correlate a particular part of the ABR to a specific stimulus pulse for the unmodified sound. The last ABR waves persisted in the middle of the stimulus. For the modified sounds consisting of two pulses, both pulses were represented by the ABR waves at hearing threshold.

Representation of frequency contents

The drumming sound stimulus of *P. pictus* showed a harmonic structure with spectral peaks (corresponding to the four harmonics h1–h4) around 200 Hz, 400 Hz, 600 Hz and 800 Hz (always ±20 Hz due to the filter bandwidth of 50 ms for the FFT calculation, which caused frequency steps of 20 Hz during analysis). Peak energies occurred within h3 and h1 (the fundamental frequency; Fig. 5A). The ABR waves evoked by this sound showed spectral peaks corresponding to h1–h4 of the stimulus and three further peaks at frequencies that would correspond to h5–h7.

The sound pulses of *B. modesta* were low-pitched, rather broadband and showed no harmonic structure. The peak energy of the two-pulsed knocking sound was around 469 Hz (Fig. 5B). By contrast, the peak spectral energy of the corresponding ABR traces was around 156 Hz. The overall shapes of both spectra did not fit to each other and no clear correspondence in spectral peaks was detected (which is also partially because broadband spectra have no clear peaks). The last pulse of the conspecific sound consisting of three pulses was also rather broadband but with its peak energy at a lower

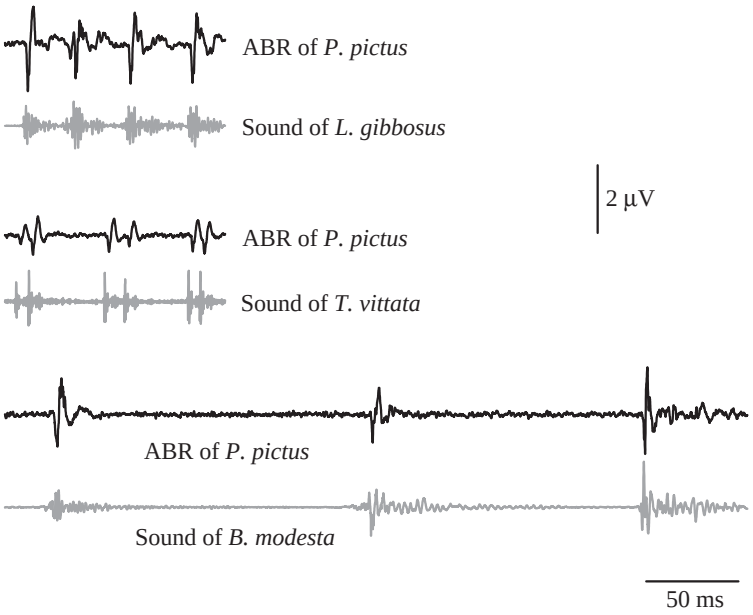

Fig. 6. Auditory brainstem response (ABR) waveforms (dark traces) of *P. pictus* in response to heterospecific sound stimuli (light traces). All sounds were played back at a sound pressure level of 100±1 dB re 1 µPa. For further details, see Fig. 2.

frequency of about 273 Hz (Fig. 5C). In this case, ABR spectrum shape more closely paralleled the stimulus spectrum, especially in the frequency range of the stimulus with the main energy content between 230 Hz and 430 Hz; both ABR spectra, however, were relatively similar to each other.

The sound pulse of *L. gibbosus* was broadband with a peak energy at 547 Hz (Fig. 5D). This peak energy corresponded to a spectral peak of the ABR. The stimulus and ABR spectra shapes were quite parallel above 400 Hz, especially between 470 Hz and 840 Hz where the stimulus had its main energy content.

Representation of heterospecific sounds

Control tests using heterospecific sounds were performed in order to test (1) whether the differences observed between *L. gibbosus* and the hearing specialists were due to the sound itself or to differences in auditory processing and (2) whether or not the fine temporal representation observed in hearing specialists was restricted to conspecific sounds. Sounds of *T. vittata*, *L. gibbosus* and *B. modesta* (three-pulsed sound) were presented at an SPL of 100±1 dB to four specimens of *P. pictus*. The catfish showed a similar temporal response pattern to heterospecific sounds (Fig. 6). The onsets of single sound pulses were highly correlated to the onsets of the first negative peaks of the corresponding ABR waves (r=1, P<0.001 for all the sounds tested). Similar to the responses to conspecific sounds, brainwave amplitudes were not correlated significantly to amplitudes of the sound pulses of *B. modesta* and of *T. vittata* (r=0.423, P=0.164; r=0.248, P=0.242, respectively). For the sound of *L. gibbosus*, a negative correlation was observed (r=–0.560, P=0.024).

In contrast to *P. pictus*, *L. gibbosus* showed irregular response patterns to the high-pitched stridulation sound of *P. pictus* and *T. vittata* (Fig. 7). Repeatable responses to these sounds could only be elicited at very high SPLs (120 dB for the sound of *T. vittata* and 129 dB for the stridulation sound of *P. pictus*) in all four individuals tested. Only one out of four individuals showed a response reflecting the temporal structure of both sounds. The responses to the sound of *B. modesta* (at an SPL of 113 dB) consisting of three pulses were more consistent among individuals. Similar to the responses to conspecific sounds, brainwaves were very long, lasting approximately 50–90 ms. As the pulse period within the sound was much longer than in the sound of *L. gibbosus*, separate responses to each of the sound pulses were detectable, and the sound's temporal structure was reflected within the ABR (r=1, P<0.001). No correlation between amplitudes was performed because it was not possible to choose standardizable and identifiable measuring points in the diverse brainwaves due to the very long responses to the sound pulses. These results show that the difference between *L. gibbosus* and the hearing specialists is not due to the conspecific sound stimulus itself and that this species has difficulty in detecting high-pitched sound.

Auditory sensitivity to conspecific sounds

Mean (±S.E.M.) hearing thresholds for conspecific sounds were 64.1±1.7 dB re 1 µPa in *P. costatus*, 62.9±0.6 dB for stridulation sounds and 75.7±1.2 dB for drumming sounds in *P. pictus* and 90.8±3.1 dB in *T. vittata* (Fig. 8). In *B. modesta*, the mean hearing threshold for the broadband sound consisting of three short pulses was 77.3±1.3 dB and that for the sound consisting of two longer pulses was 83.5±0 dB. In *L. gibbosus*, the mean hearing threshold for the natural sounds was 97.9±1.2 dB, whereas for the second test, lacking pulses 2 and 3, it was 95.7±1.9 dB.

In all hearing specialists, hearing thresholds were 26–56 dB under the minimum SPL of conspecific sounds calculated for each species from previous studies (Ladich, 1998, 1999; Wysocki and Ladich, 2001) and 41–65 dB below the averaged

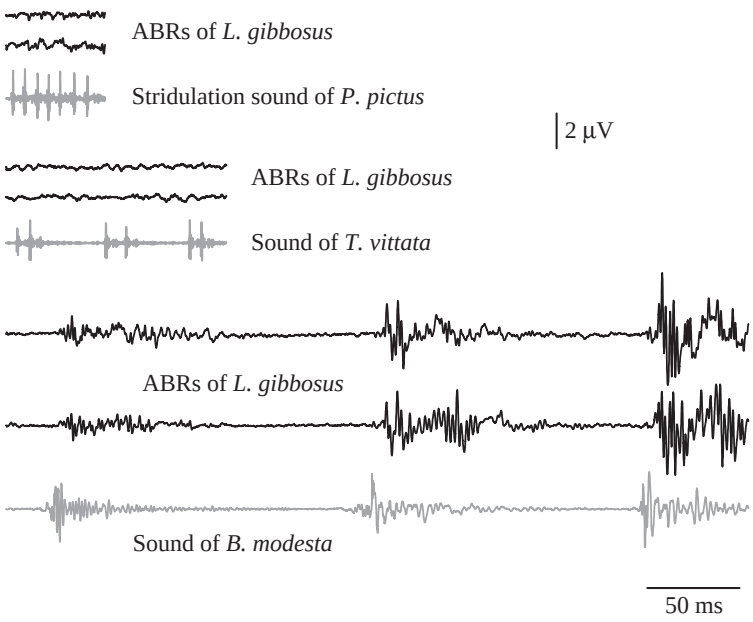

Fig. 7. Auditory brainstem response (ABR) waveforms (dark traces) of two different individuals of *L. gibbosus* in response to heterospecific sound stimuli (light traces). Sounds were played back at sound pressure levels of 113 dB (stridulation sound of *P. pictus*), 120 dB (sound of *T. vittata*) and 129 dB (sound of *B. modesta*) re 1 µPa. For further details, see Fig. 2.

SPLs of the conspecific sounds emitted by the fish (Fig. 8). No SPL measurements were available for *L. gibbosus* sounds.

Discussion

The ABR approach in investigating auditory processing of complex auditory stimuli

Pure tones, tone bursts and clicks have typically been used to investigate hearing abilities of fishes. Studies on acoustic communication have focused on how the sound spectra fit to the audiograms in terms of dominant frequencies of sounds *versus* best hearing frequencies (e.g. Ladich and Yan, 1998; Ladich, 1999). Generally, a large overlapping area between sound power spectra and audiograms was interpreted as representing sounds produced well above hearing threshold, whereas a small overlap or a lack thereof suggested that the sounds (at least at the distances and SPLs measured) are unlikely to be perceived. One case for the latter was early stages of ontogeny (Wysocki and Ladich, 2001). For other purposes, such as investigating critical bandwidths (Tavolga, 1974; Hawkins and Chapman, 1975), masking effects (Buerkle, 1969; Fay, 1974; Fay and Coombs, 1988) or other aspects of auditory processing in fishes, more complex sound stimuli were used. Such sinusoidal amplitude-modulated tones, noise bands, beats or click trains sometimes approached the characteristics of natural sounds (Bodnar and Bass, 1997; McKibben and Bass, 1998; Marvit and Crawford, 2000). The sound stimuli used in fish audiology, however, often did not reflect sounds that the animals actually confronted in their environment. To our knowledge, natural unmodified conspecific sounds have never been used as stimuli for audiological investigations in fishes. This makes our study the first to investigate directly how natural conspecific sounds are processed by the auditory system.

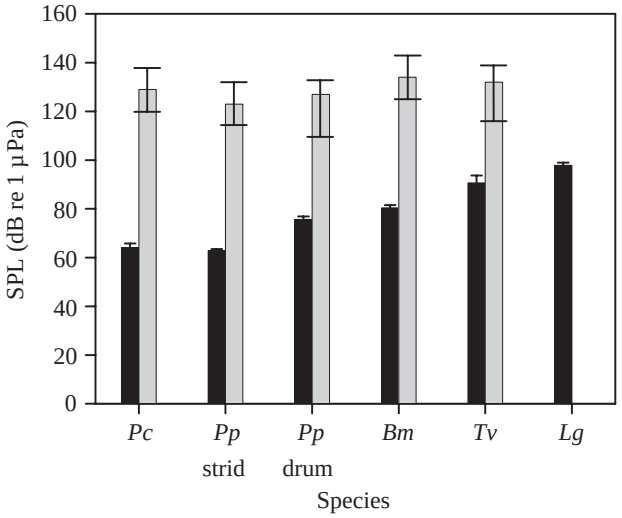

Fig. 8. Mean (± S.E.M.) hearing thresholds (dark bars) and mean sound pressure levels (SPLs; RMS fast, calculated to a distance of 3 cm from the measuring hydrophone; light bars) of the sounds produced by the different species in dB re 1 µPa. The line bars within the mean SPLs indicate the range measured from minimum to maximum SPLs. SPL values of sounds were measured during previous studies (Ladich, 1998; Ladich, 1999; Wysocki and Ladich, 2002). *Bm*, *Botia modesta* (all sounds); *Lg*, *Lepomis gibbosus* (natural sounds); *Pc*, *Platydoras costatus*; *Pp* strid, *Pimelodus pictus* stridulation; *Pp* drum, *Pimelodus pictus* drumming sounds; *Tv*, *Trichopsis vittata*.

Auditory sensitivity to conspecific sounds

The auditory sensitivity to conspecific sounds of all hearing specialists was comparable with previously measured tone burst thresholds of audiograms in the most sensitive hearing range (Ladich and Yan, 1998; Ladich, 1999). Of all the species

tested, *P. pictus* had the highest sensitivity to stridulation sounds. This high sensitivity to broadband pulses fits well to tone burst audiograms, which are quite flat in the high frequency range (500–3000 Hz; lowest hearing thresholds of about 67 dB re 1 μPa in this range; Ladich, 1999). The lower sensitivity to the low-pitched drumming sounds (approximately 13 dB less sensitive compared with the stridulation sounds) corresponds also to the lower sensitivity at lower frequencies (below 500 Hz; lowest thresholds of approximately 74 dB) revealed by the audiogram (Ladich, 1999). *B. modesta* also showed a different sensitivity to both sound stimuli tested, which were broadband and relatively low-pitched but differed in their peak frequencies (273 Hz *versus* 469 Hz) and duration of the single pulses. The pulses to which the loaches were less sensitive by about 6 dB were approximately twice as long as the pulses of the second sound tested. In ABR audiometry, short stimuli with an abrupt onset are known to be more efficient in evoking auditory potentials than stimuli with a long rising time (Hall, 1992). A single unit study on the catfish *Ictalurus nebulosus* (Plassmann, 1985) revealed the existence of two different main types of neurons in the medulla and mesencephalon, a non-adapting (tonic) type and a fast-adapting type that showed a steadily declining response with increasing stimulus rise time. Beyond frequency effects, similar response characteristics of neuron populations in the loaches might also be responsible for the different detection thresholds observed (see Fig. 2). *Trichopsis vittata* showed less sensitivity than the otophysines; this corresponds to the most sensitive frequency range in the audiogram of this species.

A comparison was made between hearing thresholds and the SPLs of sounds emitted by the hearing specialists and calculated to a similar distance to the fish (3 cm) as the calibration of the stimulus underwater. This comparison showed that even the minimum sound levels measured were at least 25 dB and up to 60 dB above the hearing thresholds of these sounds. This points to a high detectability by the fish, especially because sound communication in these species most likely occurs at short ranges.

The sunfishes showed the least sensitivity to conspecific sounds. The SPL hearing threshold of 97 dB, however, is about 20 dB more sensitive than that in response to tone bursts in the range of the main sound energies (300–600 Hz) found in the closely related species *Lepomis macrochirus* by other authors using a very similar ABR measuring technique (Scholik and Yan, 2002). Beyond species differences, this might be due to the very different type of acoustic stimuli used. As hearing generalists are not pressure sensitive, and can only detect the particle motion component of a sound, it might not seem correct to measure their sensitivity in SPLs or to make comparisons. The main goal of this study, however, was to investigate how conspecific sounds are represented in the auditory system, in particular with regard to their temporal structure in different species, and less so to determine absolute sensitivity, which we therefore will not discuss further.

Representation of temporal patterns of conspecific sounds in the brainstem of teleosts

Many natural sounds, particularly in a noisy environment such as water, are broadband but with distinct temporal patterns. This might promote the reliability of sound propagation in acoustic communication, where the spectral content of the signal is often distorted because of absorption; this variously enhances or cancels out particular frequencies depending on water depth and the pressure release surface (Parvulescu, 1964). Therefore, a broad sound spectrum is thought to guarantee the maximal acoustic signal under all water conditions (Gerald, 1971). In shallow marine waters, the most reliably propagated sound characteristic of damselfishes proved to be the pulse period (Mann and Lobel, 1997). Many fish sounds are indeed broadband pulsed sounds, and temporal patterns seem to be important factors for communication and species recognition in damselfishes, sunfishes and cods (Spanier, 1979; Gerald, 1971; Hawkins and Rasmussen, 1978).

A recent psychophysical hearing study in *Pollimyrus* (Marvit and Crawford, 2000) also concluded that the temporal discrimination abilities were sufficient not only to discriminate between the calls of its own and closely related species but most probably even between individuals. In *Pollimyrus adspersus*, some neurons of the torus semicircularis in the midbrain were found to show selective responses for a narrow range of ICIs, whereas another group of neurons was classified as non-selective within the 10–80 ms ICI range tested. The distribution of best ICIs overlapped the 5–50 ms range of communication sounds for this species (Crawford, 1997b). In all species investigated during the present study, with the exception of the hearing generalist *L. gibbosus*, each pulse within a sound elicited a separate ABR wave. Thus, independent of sound length, the temporal pattern was reflected at the level of the brainstem and is therefore a potential carrier of information in hearing specialists. However, this ability to exactly reflect temporal patterns is not restricted to conspecific sounds. In *P. pictus*, for example, the auditory system also reflected the temporal structure of heterospecific sound stimuli. In their natural environment, fishes are confronted with diverse sounds besides intraspecific communication sounds (e.g. from prey and predators) that also provide important information. Therefore, hearing abilities should not be limited to conspecific sounds. Preliminary tests showed that the auditory system of hearing specialists responded similarly to temporal patterns of heterospecific sounds. In a previous study on temporal processing of double clicks, vocal as well as non-vocal fishes showed similar temporal resolution abilities (Wysocki and Ladich, 2002). The high correlation between stimulus pulse onsets and onsets of corresponding ABR waves indicates that the auditory system closely followed the temporal structure of sounds. This implies that individual variations can be recognized. In croaking gouramis, for example, the pulse periods within a double pulse differ due to morphological asymmetries between fins, which was also the case in our sound stimulus. Such asymmetries in absolute pulse periods or

even tendencies to produce triple pulses (L. E. Wysocki, personal observation) make individuals quite differentiable.

Variations in latencies to the stimulus pulses were correlated to the pulse amplitudes of conspecific sounds. Increasing ABR latency with decreasing SPL is a common phenomenon in ABR audiometry of mammals (e.g. Supin and Popov, 1995) and fishes (e.g. Kenyon et al., 1998; Kratochvil and Ladich, 2000). The present study shows that this phenomenon also occurs within a series of sound pulses.

In the sunfish, a hearing generalist, the representation of temporal information in the brainstem was less clear. ABRs to a stimulus pulse are very long compared with specialists (several dozen ms). This might be due to fundamental differences compared with specialists either in the auditory periphery (lack of accessory hearing structures) or at more central levels of the auditory system. Testing the responses to a modified sound of only the first and the last pulse (pulse period approximately 88 ms) yielded two separate responses. Subtracting the responses to the two-pulsed sound from those to the four-pulsed sound revealed a remaining response to the mid-sound pulses. This approach was successfully applied in dolphins and fishes to determine whether an ABR waveform consisted of two responses to separate clicks that are superimposed or of only one response (Supin and Popov, 1995; Wysocki and Ladich, 2002); waveform subtraction is also a validated procedure in human ABR audiometry (Burkhard and Deegan, 1984). We can therefore assume that each of the four pulses within the tested conspecific sound stimulus contributed to the evoked response. This is supported by the findings of Gerald (1971), who showed in behavioral playback studies that sunfishes are, to a certain extent, able to selectively respond to conspecific sounds that mainly differed in their time domain. In order to test whether this difference to hearing specialists was due to differences in audition or simply to the type of sound tested, control experiments were performed using heterospecific sounds as stimuli. The responses of *L. gibbosus* to the low-pitched sound of *B. modesta* were comparable with the responses to conspecific sounds. Brainwaves lasted several dozens of ms but, because the pulse period of the sounds was long enough, three separate responses to each of the sound pulses were detectable and the temporal structure of the sounds was well represented by the auditory system. These findings agree with those obtained using the conspecific sound and its modification. By contrast, the individual sunfish showed an irregular response pattern to the high-pitched sounds of *P. pictus* and *T. vittata*. A repeatable response was obtained only at very high SPLs, and the temporal structure of the sound was only reflected clearly by one individual. We assume that only the low-frequency component of the sounds elicited the ABRs because hearing generalists have a limited hearing range, whereas stridulation sounds of *P. pictus* and croaking sounds of *T. vittata* have their main energies above 1 kHz.

Representation of intensity and spectral content of sound

In contrast to the pulse periods, the correlation between pulse amplitudes and amplitudes of the corresponding ABR wave was significant in only two out of four species and close to significance in *T. vittata*. This can mean that temporal structure, beyond sound intensity, might play a role in assessing conspecifics. Ladich (1998) showed in behavioral tests that overall sound intensity is one factor influencing the outcome of agonistic interactions in croaking gouramis. McKibben and Bass (1998) demonstrated in playbacks with tonal stimuli that female plainfin midshipman (*Porichthys notatus*) preferentially approached the more intense of two signals that differed by just 3 dB.

Other sound characteristics are certainly also important in diverse species. The dominant frequency of sounds (which is often correlated directly to body mass, depending on the sound-producing mechanism) is known to play a role during mate choice in damselfishes (Myrberg et al., 1986) and for the outcome of aggressive interactions in croaking gouramis (Ladich, 1998). In the present study, spectral comparisons between ABR components and sound stimuli were only performed for low-pitched sounds with main frequencies below 1 kHz because of the filter settings of the electrode preamplifier. The clearest result was obtained for the drumming sound of *P. pictus*, which showed a harmonic structure. The fundamental frequency of the sound was predominantly present in the ABR spectrum. In addition to the spectral peaks at h1–h4 of the stimulus, response components were consistently observed in all individuals at frequencies that presumably corresponded to h5–h7. A similar phenomenon has been observed in human ABR spectra to two-tone approximations of steady-state vowels (Krishnan, 1999). In humans, spectral peaks of the responses were not only observed at the formant frequencies of the sounds tested but also at frequencies that corresponded to harmonics of one formant of the stimulus and were not present in the stimulus spectrum. Spectral peaks within ABRs matching to spectral peaks of sound stimuli reflect temporally locked activities of populations of neurons to the frequency components of the stimuli. Neural phase-locking plays an important role in encoding spectral features of sounds (Krishnan, 2002).

In *L. gibbosus*, most similarities between stimulus and ABR spectra were found in the range of the main sound frequencies. As the sound is rather broadband, it is difficult to correlate particular spectral peaks to each other. Nonetheless, the overall spectral similarity in this particular frequency range indicates some influence of frequency components on the auditory system of the fish.

In *B. modesta*, it was not possible to interpret ABR spectral peaks as specifically representing the frequency components of the sound stimuli. However, the ABR waveforms differed in response to both sound stimuli (see Fig. 2B). This may be induced by a different activation pattern in the neurons and indicates that the perception of differences probably also relies on other characteristics such as pulse duration or envelope shape of the stimulus. There is evidence that in plainfin midshipman the different frequency of acoustic beats and the modulation frequency of amplitude-modulated signals are coded differently by neurons in the auditory midbrain, even if

it is the same frequency (Bodnar and Bass, 1997). This could permit discrimination of beats (due to concurrent vocalizations of males during the breeding season) from other amplitude-modulated like signals.

Note that ABR waves only reflect the first steps of signal processing in the brainstem up to the midbrain (Corwin, 1981) and that various response parameters change along the central auditory pathway up to 'higher' brain levels (e.g. an increase in auditory sensitivity and in transient responses to stimulus onset and offset; Feng and Schellart, 1999). We therefore conclude that, besides temporal patterns, frequency and intensity characteristics can also be transmitted by acoustic signals. Together, these provide complex information for the fish during acoustic communication.

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