

Vocal development during postnatal growth and ear morphology in a shrew that generates seismic vibrations, *Diplomesodon pulchellum*



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ABSTRACT

The ability of adult and subadult piebald shrews (*Diplomesodon pulchellum*) to produce 160 Hz seismic waves is potentially reflected in their vocal ontogeny and ear morphology. In this study, the ontogeny of call variables and body traits was examined in 11 litters of piebald shrews, in two-day intervals from birth to 22 days (subadult), and ear structure was investigated in two specimens using micro-computed tomography (micro-CT). Across ages, the call fundamental frequency (f_0) was stable in squeaks and clicks and increased steadily in screeches, representing an unusual, non-descending ontogenetic pathway of f_0 . The rate of the deep sinusoidal modulation (pulse rate) of screeches increased from 75 Hz at 3–4 days to 138 Hz at 21–22 days, probably relating to ontogenetic changes in contraction rates of the same muscles which are responsible for generating seismic vibrations. The ear reconstructions revealed that the morphologies of the middle and inner ears of the piebald shrew are very similar to those of the common shrew (*Sorex araneus*) and the lesser white-toothed shrew (*Crocidura suaveolens*), which are not known to produce seismic signals. These results suggest that piebald shrews use a mechanism other than hearing for perceiving seismic vibrations.

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1. Introduction

Some small mammals, such as golden moles (Afrosoricida: Chrysochloridae) and spalacid mole-rats (Rodentia: Spalacidae), appear to respond both to airborne sound and to seismic vibrations; the interrelationships between these sensory modalities and their potential use in communication have been of research interest for over two decades (Burda et al., 1990; Willi et al., 2006b; Mason and Narins, 2010; Bednářová et al., 2013). Shrews (Soricomorpha: Soricidae) are less well-studied, but recent investigations of the piebald shrew (*Diplomesodon pulchellum*) have revealed interesting bioacoustic parallels. Captive adult and subadult piebald shrews may vibrate the entire body when held, when lifted up in

their plastic pipe shelters or when placed on a drum membrane in behavioural experiments (Volodin et al., 2012). These vibrations, at a frequency of 160 Hz, resemble the mobile phone “vibrate” mode and are apparently produced in response to a change in the substrate under their feet. These vibrations are always produced by non-vocalizing, silent animals (Volodin et al., 2012). At the same time, adult piebald shrews produce loud screech vocalizations with a deep, repetitive, sinusoid-like frequency modulation (pulse rate), coinciding in rate with the vibrations of the body (Volodin et al., 2012). In pups, the pulse rate of screeches is much lower than in adults (Volodin et al., 2015b). Body vibrations of 160 Hz are documented in piebald shrews from 34 days post-partum (Volodin et al., 2012), but have not been investigated in pups, so it is not known whether the screech pulse-rate increase, from pups to adults, is reflected in the ontogeny of body vibration too.

Piebald shrews represent a convenient model for studying vocal ontogeny, because the same call types and call variables can be measured across ages (Volodin et al., 2015b), and because of their rapid growth (Zaytseva et al., 2013). In mammals, body size and body mass increase progressively during ontogeny (Gaillard et al., 1997), whereas pathways of vocal ontogeny differ across species,

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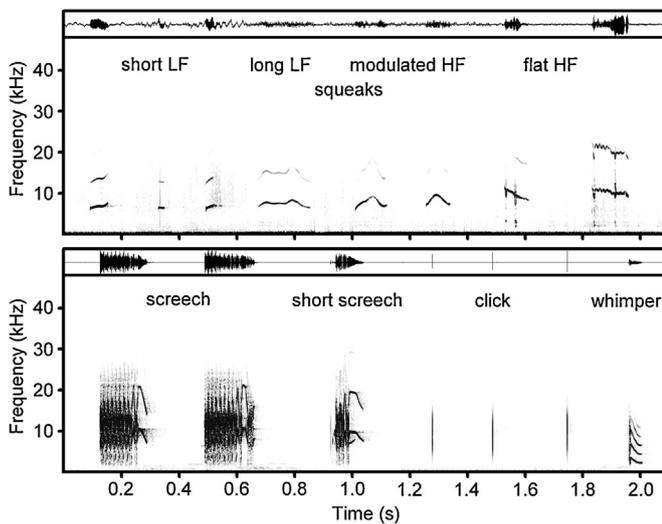


Fig. 1. Spectrogram illustrating eight call types of piebald shrew pups at 5 days of age. The spectrograms were created with a Hamming window, 48 kHz sampling rate, FFT 1024 points, frame 50% and overlap 87.5%. The audio files with these calls are provided in Supplementary material 2.

call types and call variables (Ey et al., 2007; Matrosova et al., 2007). The fundamental frequency (f_0) is typically higher in juvenile mammals than in adults (Morton, 1977). This is because acoustic differences between juveniles and adults primarily result from the differences in sizes of sound-producing structures (Fitch and Hauser, 2002). In humans and many other mammals, the f_0 is inversely related to mass and length of the oscillating portions of the vocal folds (Titze, 1994), and both mass and length increase together with the growth of the larynx (Kahane, 1978, 1982). In most mammalian species, the growth of these sound-producing structures is related to the growth of the body, which results in a steady descent of f_0 with age (for instance, Briefer and McElligott, 2011; Efremova et al., 2011; Campbell et al., 2014). In humans, this pattern is complicated in males by an abrupt fall of f_0 due to accelerated growth of the larynx at puberty (Fitch and Giedd, 1999; Lee et al., 1999). Nevertheless, in a few species of ground squirrels, the f_0 s of alarm calls are indistinguishable between pups and adults, in spite of much larger bodies and larynges in adults than in pups (Matrosova et al., 2007; Swan and Hare, 2008; Volodina et al., 2010). The f_0 also increases with body growth in bat pups, in both echolocation calls and social calls (Jones et al., 1991; Hiryu and Riquimaroux, 2011; Jin et al., 2011, 2012).

An ontogenetic study of body traits and body mass in 18 litters of piebald shrews demonstrated that they grow very rapidly (Zaytseva et al., 2013), similarly to other shrews (Dryden, 1968; Vlasák, 1972; Michalak, 1987). In piebald shrews, weaning is at about 20 days of age (Vakhrusheva and Ilchenko, 1995) and first copulations were recorded at 27 days in females and at 40 days in males (Ilchenko et al., 2011). At separation from the mother at 22 days, shrews are comparable in body length to reproductively mature adults (62 mm and 70 mm, respectively, Zaytseva et al., 2013).

In captivity, piebald shrews produce eight call types, all within the human audible frequency range (<20 kHz): short and long low-frequency squeaks with nearly flat contour, high-frequency squeaks with modulated contour, high-frequency squeaks with fractured contour, short screeches and screeches, clicks and whimpers (Fig. 1). Seven of the eight call types are shared by pups and adults, suggesting that this vocal repertoire is established at birth (Volodin et al., 2015b). The previous cross-sectional study revealed that the f_0 of all four types of squeaks were indistinguishable between pups and adults, whereas the f_0 of screeches was higher in adults than in pups (Volodin et al., 2015b). However, the rate of

deep sinusoidal frequency modulation (hereafter “pulse rate”) of screeches was found to be substantially and significantly lower in pups than in adult piebald shrews. While an ontogenetic study of body features and a cross-sectional study of call types and call variables already exist, an ontogenetic study of the acoustics is lacking for this species.

Body vibrations are produced by silent piebald shrews, independent of any vocalizations (Volodin et al., 2012). Perhaps they generate these vibrations for communication purposes, for example as a warning aimed either at conspecifics or potential predators. Alternatively, they might use ‘seismic echolocation’ for spatial orientation purposes, analogous to what has been proposed for subterranean *Spalax* mole-rats (Kimchi et al., 2005). During the night, piebald shrews patrol their semidesert habitats and dig up invertebrates from depths of 2–3 cm in sand (Dubrovskij et al., 2011), so it is possible that they detect reflected vibrations from substrate heterogeneities, such as those related to the presence of their prey. Hypotheses relating to intraspecific communication and seismic echolocation demand that the shrews have a means of detecting seismic vibrations. In principle, seismic vibrations could be detected following radiation into the air, resulting in airborne sound which is detected by the ear in the normal way. Alternatively, vibrations could be detected directly if the body or head is in contact with the vibrating substrate, by somatosensory receptors or by a form of bone-conducted hearing (see Mason and Narins, 2010 for a review). Bone-conducted hearing may be the sensory modality employed by golden moles, in which the hypertrophied mallei may be used as inertial sensors (Mason, 2003a,b; Willi et al., 2006a); some unusual features of the ear of the mole-rat *Spalax ehrenbergi* have also been interpreted as adaptations to promote bone conduction (Rado et al., 1989), although this view has been challenged (Mason et al., 2010). Although piebald shrews are not subterranean mammals, their ability to produce seismic vibrations might similarly be reflected in their ear morphology: this possibility has not been investigated previously.

The general focus of this study was to consider the relationship between vocal and body ontogeny, and to explore whether functional associations exist between vocalizations, vibration generation and ear morphology. The particular aims were (1) to define the relationships between changes in acoustic variables, body mass and head length during the ontogeny of piebald shrews, and (2) to examine the ear morphology to see if there are any obvious adaptations promoting vibratory sensitivity.

2. Materials and methods

2.1. Study site and subjects

Calls as well as measurements of body mass and head length were collected from members of a captive colony of piebald shrews at Moscow Zoo, Moscow, Russia, from 1 June to 22 August 2011. Our live subjects were 40 piebald shrews (24 males and 16 females from 11 litters) examined from birth to separation from the mother at 22 days of age. All study animals (3rd–6th generations in captivity) were descendants of 27 animals collected in 2008 in the Astrakhan Region, Russia (47°12′33″N; 48°18′45″E).

The animals were kept under a natural light regime at room temperature (24–26 °C), in family groups consisting of a mother and littermates. The animals were housed in plastic cages of 53 × 76 × 42 cm, with a bedding of sand and dry moss, various shelters and running wheels. They received custom-made small insectivore chow with insect and calcium supplements, and water *ad libitum*. Before parturition, females were checked twice a day for the appearance of a litter, and birth dates as well as the number of pups were recorded. Litter size varied from 3 to 6 pups

(mean \pm SD = 3.9 ± 1.1), with 43 pups in total being born. Three pups died at 1 or 2 days of age. The registered first day post-partum was considered to be the first day of pup life for the chronological splitting of age groups along ontogeny. The 11 study litters originated from 11 different mothers. For each mother, the study litter was her first litter in the breeding season. Study pups were sexed between 3 and 9 days of age based on the appearance of nipples in females (Vakhrusheva and Ilchenko, 2010). The small size of pups during the study period (the mean body mass of a 5 day-old pup was 2.83 g and body length was 38.5 mm: Zaytseva et al., 2013) prevented individual marking for ethical reasons. The definitive pinnae appear only at day 9–10 of pup life (Zaytseva et al., 2013), so newborn piebald shrews could not be individually marked by cuts on ears.

2.2. Call and body growth data collection

Calls were recorded collectively from all pups of each litter (*i.e.* the calling pups within a litter were not identified individually), whereas body mass and head length measurements were taken from each littermate separately. Pups were weighed and measured for the first time on the first post-partum day of their lives, if they were born in the morning or during the day, or otherwise on the second day. After that, body mass and head length measurements were taken every two days up to the age of 22 days, when pups were separated from the mother. For weighing, we used G&G TS-100 electronic scales (G&G GmbH, Neuss, Germany), accurate to 0.01 g. For head length measurements, we used electronic calipers (Kraf Tool Co., Lenexa, Kansas, US), accurate to 0.01 mm. When making these measurements, the litter was separated from the mother and returned within 40 min. Mothers were tolerant of the temporary absence of their pups and no pup appeared to suffer from this experience. Pups younger than 10 days were placed for weighing directly on the scales; pups older than 10 days were placed for weighing in a transparent plastic can. After weighing, we measured head length of the hand-held pup from the tip of the snout to the occiput. This measurement was repeated three times and the mean value was taken for analysis.

Pup calls were recorded from individuals separated from their mother and placed on a clean, smooth table-top area (60×60 cm) or in a clean plastic enclosure ($28 \times 43 \times 14$ cm), depending on the age. In addition, some calls were recorded when pups were together with the mother (primarily during caravanning at 12–16 days of age). Caravanning represents a behaviour whereby a pup catches the fur at the base of the mother's tail, and then the rest of the litter attaches one to another in the same way (Vlasák, 1972; Tsuji and Ishikawa, 1984). Caravanning could only be elicited in the presence of the mother (Schneiderová, 2014).

Acoustic recordings were made on the day of weighing, but at least 5 h after the individuals were weighed. All acoustic recordings were conducted in a separate room where no other animals were present, at room temperature ($24\text{--}26^\circ\text{C}$) during daytime. For sound recordings (sampling rate 96 kHz, 24 bit resolution) we used a Fostex FR-2LE professional digital recorder (Fostex Company, Tokyo, Japan) and a Sennheiser K6-ME64 condenser microphone (Sennheiser electronic, Wedemark, Germany). In addition, to monitor the potential presence of ultrasound, half of the recordings were made also in the ultrasonic range with sampling rates of 192 kHz or 768 kHz, 16 bit resolution, using a Pettersson D 1000X recorder with built-in microphone (Pettersson Elektronik AB, Uppsala, Sweden). All microphones were kept at distance of 5–15 cm from the animals. Each trial lasted 7–14 min and was recorded as a wav-file.

2.3. Call samples

We chronologically subdivided acoustic recordings made from the 11 litters into 11 age-groups (hereafter “ages”) of 1–2, 3–4,

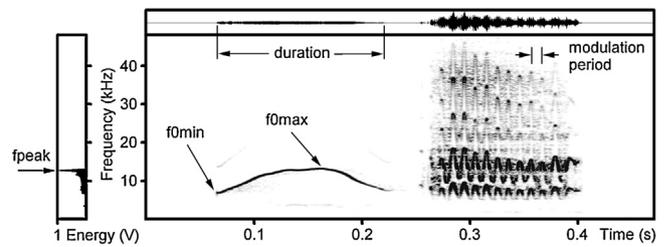


Fig. 2. The measured acoustic variables in (left) power spectrum and spectrogram with waveform (right). Designations: duration – call duration, modulation period – period of the deep sinusoidal frequency modulation, then used for calculating “pulse rate” of screeches and short screeches; $f_{0\max}$ – maximum frequency of f_0 , $f_{0\min}$ – minimum frequency of f_0 , f_{peak} – maximum amplitude frequency. The spectrogram was created with a Hamming window, 48 kHz sampling rate, FFT 1024 points, frame 50% and overlap 96.87%.

5–6, 7–8, 9–10, 11–12, 13–14, 15–16, 17–18, 19–20 and 21–22 days post-partum. This provided matched data on the vocalizations, body mass and head length measurements during the development of each litter. The percentages of recording trials in which a given call type was emitted were used as measures of the occurrence of different call types, for pups at different ages. For some litters at some ages, recording trials were missing (due to delayed detection of the litter or for other reasons). As a result, only 105 trials (81 without mother and 24 with mother) of the potential 121 recording trials were made, and therefore from 7 to 11 recording trials per age were available for acoustic analyses of 7 call types (Volodin et al., 2015b).

If calls of the given type were emitted in the given recording trial, we took for acoustic analysis 1–20 calls (mean \pm SD = 7.00 ± 5.62 ; $n = 464$) per call type (excluding whimper) per recording trial: 3095 calls in total. If the number of calls per trial of a particular type was ≤ 20 , all available calls were included in the analysis; if it was > 20 , we selected 20 calls per call type per trial for analysis, taking calls from different parts of a trial, arbitrary, to decrease pseudoreplication. When this was impossible, we took calls separated by at least one call of another type. In total, we selected for analysis 1270 short LF squeaks, 303 long LF squeaks, 568 modulated HF squeaks, 256 flat HF squeaks, 323 screeches, 110 short screeches and 265 clicks (Fig. 1). We excluded the whimper from acoustic analyses, as this call type was made only within a very short period of development, from the second to the sixth day post-partum (Volodin et al., 2015b).

2.4. Call analysis

Inspection of spectrograms of the acoustic files recorded in the ultrasonic range (> 20 kHz), using Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany), did not reveal any ultrasonic components. All spectrographic analyses were performed with Avisoft SASLab Pro (sampling frequency 96 kHz) and the results were exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA). As minimum fundamental frequency ($f_{0\min}$) of calls always exceeded 1 kHz (Volodin et al., 2015b), before measurements all wav-files were subjected to 1 kHz high-pass filtering and 30 kHz low-pass filtering, to remove low-frequency noise and to avoid aliasing (high-frequency aberrations), respectively. For all call types, we measured the duration and the frequency of maximum amplitude (f_{peak}) from the call's mean power spectrum, using the option “Automatic parameter measurements” in Avisoft (Fig. 2). For all call types excluding clicks, we measured the maximum fundamental frequency ($f_{0\max}$) with the reticule cursor in the spectrogram window (sampling frequency 96 kHz, Hamming window, FFT 1024 points, frame 50%, overlap 96.87%, providing frequency resolution 93 Hz and time resolution 0.3 ms) (Fig. 2). For screeches

and short screeches, we additionally measured the mean rate of the deep sinusoidal frequency modulation (thereafter “pulse rate”) as the inverse value of the mean modulation period, using the standard marker cursor in the main window of Avisoft (Fig. 2). For clicks, we measured the click fundamental frequency as the inverse value of the mean f_0 period, visible on the extended (zoomed-in) waveform, using the standard marker cursor in the main window (for details, see Volodin et al., 2015b). This f_0 value was used as $f_{0\max}$ of clicks in subsequent statistical analyses.

2.5. Anatomical specimens and ear scans

CT-scans of two *Diplomesodon* specimens, which died by accident in the study colony of the Moscow Zoo, were made at the Berlin Museum of Natural History. These specimens were a subadult female (body mass 7.1 g) and an adult male (body mass 17.2 g). The head was not prepared in any way: the entire animal was in each case placed into a General Electric Phoenix Nanotom X-ray Micro-CT scanner at the Museum für Naturkunde, Berlin. The scanner was operating at 80–90 kV and 150–350 μ A. 800 or 1400 projections were taken over 360° total rotation, each with 750 msec exposure and 3 frames averaged per projection. Voxel linear dimensions were 6–8 μ m for close-ups of the head, or 30 μ m for whole-body scans. Scan data were processed using Datas|x software (GE Sensing & Inspection Technologies GmbH – phoenix|X-ray).

For comparative analyses of ear morphology, the ear regions of two other shrew species were examined. Two specimens of *Sorex araneus* (body mass 5.9 and 7.3 g) were found as corpses in Cambridgeshire, U.K.; one specimen of *Crocidura suaveolens* (body mass 5.4 g) was found as a corpse on Gugh, Isles of Scilly, U.K. These shrews were probably killed by cats. One *Sorex* and the *Crocidura* specimen were preserved by freezing prior to examination while the other *Sorex* was fresh. The heads of all of these animals were removed, skinned, covered in cellophane to reduce the rate of drying and taken for CT scanning. The ear region of one of the *Sorex* specimens was later dissected out, allowed to dry and scanned again at higher magnification. The CT-scans of the *Sorex* and *Crocidura* specimens were made using a Nikon XT H 225 scanner at the University of Cambridge. The scanner was operating at 106–120 kV and 148–183 μ A for the whole head scans, or at 79 kV and 246 μ A for the auditory region scan. 720 or 1080 projections were taken over 360° total rotation, each with 1000 msec exposure and 1 or 2 frames averaged per projection. Voxel linear dimensions were 8–12 μ m. The software used in the processing of the scan data included CT Agent XT 3.1.9 and CT Pro 3D XT 3.1.9 (Nikon Metrology 2013, Tring, UK).

Exported tiff stacks from the scans were converted to jpg files using IrfanView 4.37 (www.irfanview.com/). MicroView 2.1.2 (GE Healthcare, 2000–2006) was used to visualise skull structure for orientation purposes, while WinSurf 4.0 (www.surfdriver.com/) was used to construct three-dimensional images of the ear, following visual identification and tracing of the borders of relevant structures. The inner walls of the bony labyrinth were traced to make the reconstructions of the inner ear.

2.6. Statistics

Statistical analyses were conducted using STATISTICA (StatSoft, Tulsa, OK, USA). Means are presented as mean \pm SD, all tests were two-tailed and differences were considered significant whenever $p < 0.05$. Distributions of all measured parameter values did not depart from normality (Kolmogorov–Smirnov test, $p > 0.05$). We used the General Linear Model (GLM) to compare the effects of age, sex and litter identity on body mass and head length. Because body mass should, under the assumption of isometry, be proportional to the cube of a linear dimension like body length, we used log

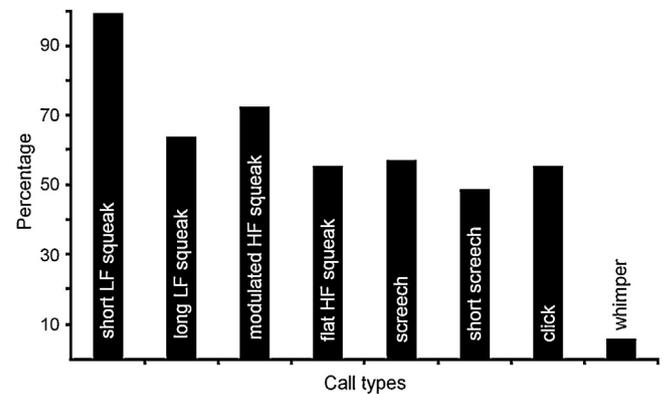


Fig. 3. Percent of recording trials containing the given call type ($n = 105$ recording trials in total across all ages).

body mass for the analysis. We calculated Pearson correlation coefficients with Bonferroni corrections to examine the correlations between body mass, head length, age and the acoustic variables (using mean values of call variables per litter per age-group). Because some litters did not produce some call types at some ages, not every litter was represented at every age-group. Therefore, we used a two-factor General Linear Mixed Model (GLMM), with age as fixed factor and the litter identity as the random factor, appropriate for analysis of data with missing values, to compare the effect of age on acoustic structure. For the analysis with GLMM, we used the mean values of each acoustic variable for each litter in each age-group. This decreased the number of degrees of freedom and allowed a more robust examination of the effects of age.

We provided effect size statistics to measure the strength of an effect in addition to statistical significance. We calculated the effect size for GLM using η^2 ($\eta^2 = 0.01$ for a small effect, 0.06 for a medium effect and 0.14 for a large effect; Cohen, 1992; Fritz et al., 2012). For the correlation analyses, the correlation coefficient itself was an estimation of the effect size ($r = 0.1$ for a small effect, 0.3 for a medium effect and 0.5 for a large effect; Fritz et al., 2012).

3. Results

3.1. Age effects on call type occurrence

Seven call types (all four types of squeaks, both types of screeches and the clicks) were detected among pups of all 11 litters, whereas the whimpers were detected in just 3 of the 11 litters. Short low-frequency (LF) squeaks were emitted in most recording trials (99.0%) (Fig. 3); next in order of occurrence were the modulated high-frequency (HF) squeaks (72.4% of recording trials) and then the long LF squeaks (63.8% of recording trials). Flat HF squeaks, screeches, short screeches and clicks were present in 48.6% to 57.1% of recording trials. Whimpers were detected in only 5.7% of recording trials.

Analysis of percentages of recording trials containing the given call type, when separated by age-group (Fig. 4), revealed that short LF squeaks were emitted at high rates (from 88.9 to 100% of recording trials) at all ages. The percentage of trials containing long LF squeaks increased steadily to 5–6 days (up to 80% of recording trials), remained at this level to 15–16 days and then abruptly fell. The percentage of trials containing modulated HF squeaks was high from birth to 17–18 days (57.1–100% of recording trials), but then fell abruptly to zero at 21–22 days. Notably, flat HF squeaks were relatively rare before 11–12 days, then frequent up to 17–18 days (70.0–81.8% of recording trials), and then became rare again.

Screeches appeared only at 3–4 days of age. The percentage of trials with screeches increased steadily up to the maximum of 90.0%

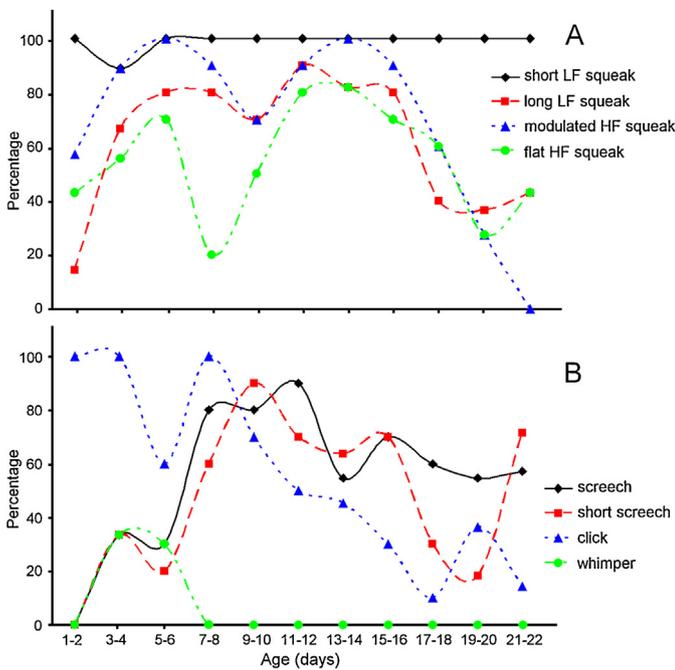


Fig. 4. Percent of recording trials containing the given call type in each age: (A) four types of squeaks; (B) screeches, short screeches, clicks and whimpers.

at 11–12 days and then decreased, varying from 54.5% to 70% of recording trials (Fig. 4). The occurrence of short screeches matched those of screeches. They also appeared at 3–4 days, increased steadily to 10–12 days, then steadily decreased, but rose up again to 71.4% at 21–22 days of age. Clicks were most frequent at early ages, but the percentage of recording trials with clicks decreased towards maturation, so after 11–12 days of age, clicks were detected in less than 50% of recording trials. Whimpers occurred very rarely and only in 4–6-day pups.

Overall, squeaks were found more often in recording trials compared to screeches and clicks. The use of screeches increased to 10–12 days of life and then decreased, whereas clicks were most frequent at early ages and showed a steady decrease in occurrence with advancing age.

3.2. Age and sex effects on body mass and head length

GLM revealed strong and significant effects of age ($F_{9,318} = 310.3$; $p < 0.001$; $\eta^2 = 0.90$) and litter identity ($F_{10,318} = 56.2$; $p < 0.001$; $\eta^2 = 0.64$), but not pup sex ($F_{1,318} = 3.6$; $p = 0.06$; $\eta^2 = 0.01$) on body mass. GLM similarly revealed strong and significant effects of age ($F_{9,318} = 352.9$; $p < 0.001$; $\eta^2 = 0.91$) and litter identity ($F_{10,318} = 22.9$; $p < 0.001$; $\eta^2 = 0.42$), but not pup sex ($F_{1,318} = 0.01$; $p = 0.95$; $\eta^2 < 0.001$) on head length. As sex effects on body mass and head length were not significant, we could use a pooled sample of pups of both sexes and operate with mean values of body mass and head length per litter for each age. Body mass and head length both increased significantly with age ($r = 0.916$, $p < 0.001$ and $r = 0.912$, $p < 0.001$, respectively) (Fig. 5).

3.3. Age effects on acoustic variables

The age effects on acoustic variables are presented for four call types (short LF squeaks, modulated HF squeaks, screeches and clicks), as these four calls are produced most frequently and are representative of the four main structural patterns (Table 1 and Fig. 6). For the remaining three call types (long LF squeaks, flat HF squeaks and short screeches), age effects on the acoustics are

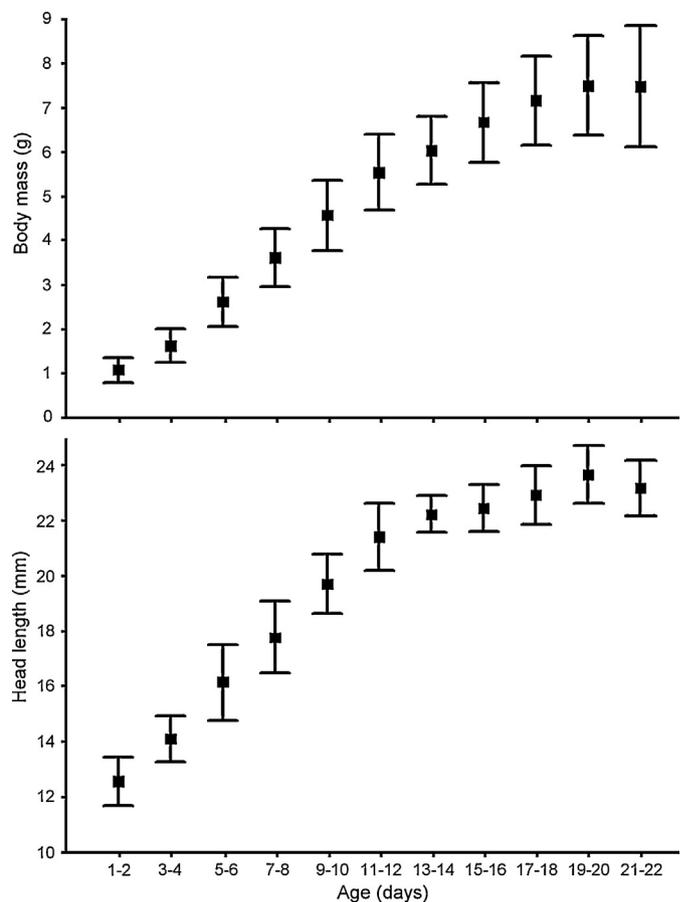


Fig. 5. Age-related changes in (A) body mass and (B) head length ($n = 8$ for 1–2 days, $n = 10$ for 3–4 days, and $n = 11$ for all other ages). Central points show means, whiskers show SD.

presented in Supplementary Material 1. Descriptive statistics of acoustic variables for all the seven call types are also presented in Supplementary Material 1.

For short LF squeaks, GLMM revealed a significant effect of age on f_{peak} , but not on f_0 or duration (Table 1). Duration of LF squeaks showed a weak although significant decrease with age, whereas f_{peak} increased significantly up to 15–20 days of age and then significantly and noticeably decreased (Table 1 and Fig. 6). The f_0 and f_{peak} of modulated HF squeaks increased with age weakly although significantly, whereas the duration was not related to pup age (Table 1 and Fig. 6). For screeches, GLMM and Pearson's correlation revealed a strong and significant increase of f_0 and f_{peak} from 3 to 4 to 13–14 days of age, followed by a plateau. The duration of screeches decreased significantly up to 13–14 days of age, also followed by a plateau (Table 1 and Fig. 6). The pulse rate of screeches steadily and significantly increased with age ($F_{9,41} = 30.54$, $p < 0.001$; $\eta^2 = 0.87$; $r = 0.884$, $p < 0.001$), from 74.8 ± 13.4 Hz at 3–4 days to 138.4 ± 11.3 Hz at 21–22 days of age (Fig. 7). For clicks, the f_0 was not related to pup age, whereas the duration significantly (although inconsistently) changed with age, and f_{peak} weakly, although significantly, increased with age (Table 1 and Fig. 6).

3.4. Body mass and head length effects on acoustic variables

As body mass and head length are positively and highly significantly correlated with age (Fig. 5), correlation coefficients of acoustic variables with body mass and head length (Table 2) were similar to correlation coefficients of acoustic variables with age

Table 1

Results of two-factor GLMM (with age as a fixed factor and the identity of a litter as the random factor) and Pearson correlation coefficients for age effects on variables of four call types, during the ontogeny of piebald shrews. Key: f_{0max} = maximum fundamental frequency; duration = call duration; f_{peak} = maximum amplitude frequency. Significant differences (for Pearson correlations after Bonferroni correction at $p < 0.016$) are shown in bold.

Call type	f_{0max}		Duration		f_{peak}	
	GLMM	Correlation	GLMM	Correlation	GLMM	Correlation
Short LF squeak	$F_{10,82} = 1.91$ $p = 0.06$ $\eta^2 = 0.19$	$r = -0.196$ $p = 0.047$	$F_{10,82} = 1.93$ $p = 0.052$ $\eta^2 = 0.19$	$r = -0.243$ $p = 0.013$	$F_{10,82} = 2.37$ $p < 0.05$ $\eta^2 = 0.22$	$r = 0.262$ $p = 0.008$
Modulated HF squeak	$F_{9,55} = 1.70$ $p = 0.11$ $\eta^2 = 0.22$	$r = 0.298$ $p = 0.010$	$F_{9,55} = 2.05$ $p = 0.051$ $\eta^2 = 0.25$	$r = 0.026$ $p = 0.83$	$F_{9,55} = 2.68$ $p < 0.05$ $\eta^2 = 0.31$	$r = 0.424$ $p < 0.001$
Screech	$F_{9,41} = 11.92$ $p < 0.001$ $\eta^2 = 0.72$	$r = 0.727$ $p < 0.001$	$F_{9,41} = 2.50$ $p < 0.05$ $\eta^2 = 0.35$	$r = -0.366$ $p = 0.004$	$F_{9,41} = 5.18$ $p < 0.001$ $\eta^2 = 0.53$	$r = 0.620$ $p < 0.001$
Click	$F_{10,34} = 1.30$ $p = 0.27$ $\eta^2 = 0.28$	$r = 0.306$ $p = 0.023$	$F_{10,34} = 3.80$ $p < 0.01$ $\eta^2 = 0.53$	$r = 0.077$ $p = 0.58$	$F_{10,34} = 1.55$ $p = 0.16$ $\eta^2 = 0.31$	$r = 0.466$ $p < 0.001$

(Table 1). The maximum f_0 was significantly positively correlated with log body mass and head length for screeches but only with head length for modulated HF squeaks. The durations of squeaks and clicks did not show significant relationships with body size, and only the duration of screeches showed a significant decrease with increasing log body mass and head length. However, in all four call types, f_{peak} increased significantly with increasing pup log body mass and head length (Table 2). The pulse rate of screeches was significantly positively correlated with log body mass and head length ($r = 0.816, p < 0.001$ and $r = 0.813, p < 0.001$, respectively).

3.5. Ear structure

CT reconstructions showed that all three shrew species examined (*D. pulchellum*, *C. suaveolens* and *S. araneus*) lack bony bullae: the tympanic cavity rostral to the cochlea is separated from the cranial cavity above only by a thin layer of fibrous tissue, and the ectotympanic bone, which supports the tympanic membrane, takes the form of an incomplete ring which is not fused to the surrounding bones. The middle ear bones and bony labyrinth of *Diplomesodon*, *Crociodura* and *Sorex* are very similar in shape and size (Fig. 8); the

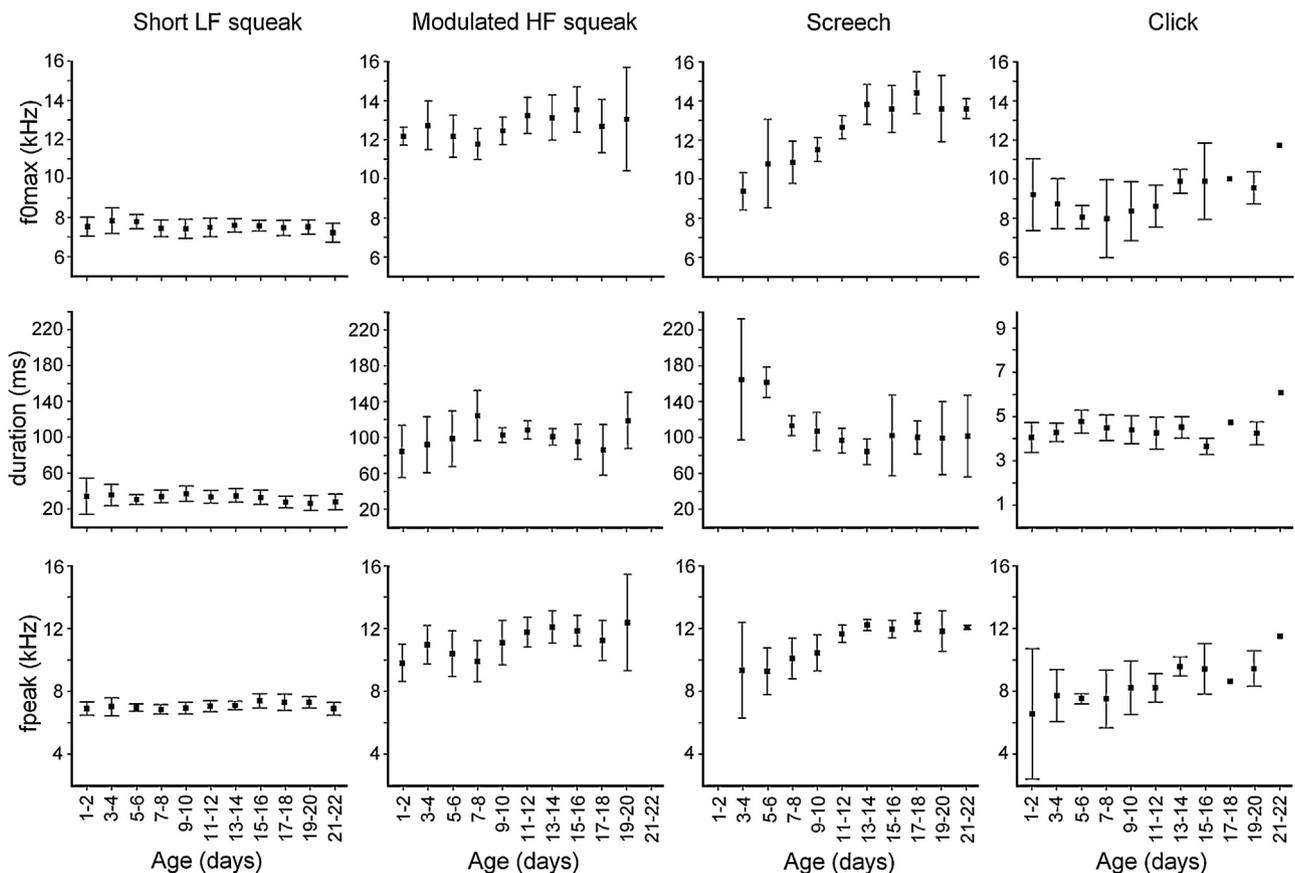


Fig. 6. Age-related changes in the maximum fundamental frequency (f_{0max}), call duration (duration) and peak frequency (f_{peak}) of four call types (short LF squeak, modulated HF squeak, screech and click) during the ontogeny of piebald shrews. Central points show means of individual litter means, whiskers show SD. Note that click durations are nearly ten times shorter than those in the other call types.

Table 2
Pearson's correlation coefficients (r) for log body mass, head length and variables of four call types of piebald shrews. N – number of mean values of call variables per litter per age-group. Correlations considered significant after Bonferroni correction at $p < 0.016$ ($0.05/3$) are shown in bold.

Call type	N	$f_{0\max}$		Duration		f_{peak}	
		Body mass	Head length	Body mass	Head length	Body mass	Head length
Short LF squeak	103	$r = -0.174$ $p = 0.08$	$r = -0.172$ $p = 0.08$	$r = -0.155$ $p = 0.12$	$r = -0.165$ $p = 0.10$	$r = 0.267$ $p = 0.006$	$r = 0.315$ $p = 0.001$
Modulated HF squeak	75	$r = 0.256$ $p = 0.026$	$r = 0.295$ $p = 0.010$	$r = 0.155$ $p = 0.18$	$r = 0.086$ $p = 0.46$	$r = 0.402$ $p < 0.001$	$r = 0.447$ $p < 0.001$
Screech	61	$r = 0.713$ $p < 0.001$	$r = 0.749$ $p < 0.001$	$r = -0.402$ $p = 0.001$	$r = -0.397$ $p = 0.002$	$r = 0.662$ $p < 0.001$	$r = 0.706$ $p < 0.001$
Click	55	$r = 0.175$ $p = 0.20$	$r = 0.229$ $p = 0.09$	$r = 0.086$ $p = 0.53$	$r = 0.092$ $p = 0.50$	$r = 0.439$ $p < 0.001$	$r = 0.430$ $p = 0.001$

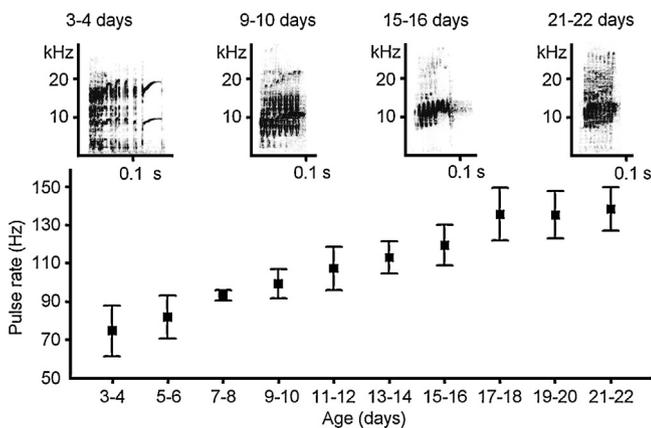


Fig. 7. Age-related changes in the rate of the deep sinusoidal frequency modulation ("pulse rate") of screeches. Central points show means of individual litter means, whiskers show SD. Representative spectrograms (with time on x-axis and frequency on y-axis) for four selected ages are presented above the graph.

adult male *Diplomesodon* specimen had ear structures which were only slightly larger than those of the subadult female. The ossicles of all three shrew species are of a 'microtype' morphology featuring a large orbicular apophysis of the malleus (bulkiest in *Sorex*), a long anterior process and a thin manubrium which is roughly parallel to the anterior process. The anterior process runs in a groove in the ectotympanic bone and may be synostosed to that bone towards its tip. In *Sorex*, the malleus and incus were indistinguishably fused, but this was not the case in either *Diplomesodon* or *Crocidura*.

The gross morphology of the inner ear is also very similar in all shrews examined. The cochlea forms a short coil. The oval window accommodates the small, elongated stapes footplate; the much larger round window is found within a deep but narrow fossula fenestrae cochleae, the entrance to which is just caudomedial to the stapes. The anterior semicircular canal is longer than the other two canals. In *Diplomesodon* and *Crocidura*, this semicircular canal lies in a plane approximately perpendicular to the ectotympanic bone, whereas the angle between the two structures is greater in *Sorex*.

4. Discussion

4.1. Vocal ontogeny with body growth

Juveniles produced eight call types, all in the human audible frequency range. All call types were produced in the same context of separation from the mother and from the nest. In addition, some calls were also recorded during the caravanning behaviour displayed by piebald shrews at 12–16 days post-partum. None of

these call types showed an ontogenetic decrease of fundamental frequency, as would be expected among mammals in general (Morton, 1977; Fitch and Hauser, 2002). The pulse rate of screeches steadily increased and became more regular with age.

Of the eight call types recorded in piebald shrews, six call types (four types of squeaks, clicks and whimpers) were made from birth, whereas screeches and short screeches were only registered from 3 to 4 days post-partum. Seven of the call types found in the young (the exception being the rarely-occurring whimper) were found also in adult piebald shrews (Volodin et al., 2015b). This is different from the ontogeny of the vocal repertoire in the Asian house shrew *Suncus murinus*, in which some call types disappear and other call types appear during postnatal development (Schneiderová, 2014). Only five juvenile call types of Asian house shrews persisted among adults: two call types disappeared and 10 call types appeared anew. In the piebald shrew, in contrast, vocal development does not involve changes in the overall set of call types, but there are stepwise changes in their structural characteristics.

The use of different call types by piebald shrews differed according to postnatal age. The study animals mainly used various squeaks. The occurrence of long LF squeaks was maximal from 5 to 6 to 15–16 days, which coincides approximately with the time of opening of ears and eyes and the development of caravanning behaviour in this species (Zaytseva et al., 2013). In adult piebald shrews, the long LF squeaks are often recorded when a male chases a female during courtship, a behaviour which is followed by copulation (our unpublished observations). It is practically impossible to identify an individual caller during courtship and sexual interaction, so it is unclear whether the male or the female is producing these calls. Similar changes in the context of calls of adults were found in Asian house shrews. Chirps of pups and adolescents of this species when caravanning did not differ in structure from adult male chirps made when courting females (Schneiderová, 2014). In an earlier study of Asian house shrews, Gould (1969) considered that chirps at courting were produced by receptive females, not by males.

Screeches and short screeches were recorded only from the 3rd day post-partum in piebald shrews. These were the only call types that were not emitted from birth. The structure of screeches of 3–4 day old pups appeared imperfect, with breaks in the sinusoidal frequency modulation which was also considerably slower than in adults, showing an irregular pulse rate. With increasing age, the pulse rate steadily increased from 75 Hz at 3–4 days to 138 Hz at 21–22 days and became more and more regular. It may well be that the maximum contraction rate of certain body muscles, involved in the deep sinusoidal modulation of their screeches, increases with postnatal age. In a study of the greater white-toothed shrew *Crocidura russula* and the Etruscan shrew *Suncus etruscus*, the deep sinusoidal modulation of screeches was found to slow progressively with cooling, leading to torpor (Hutterer et al., 1979), which

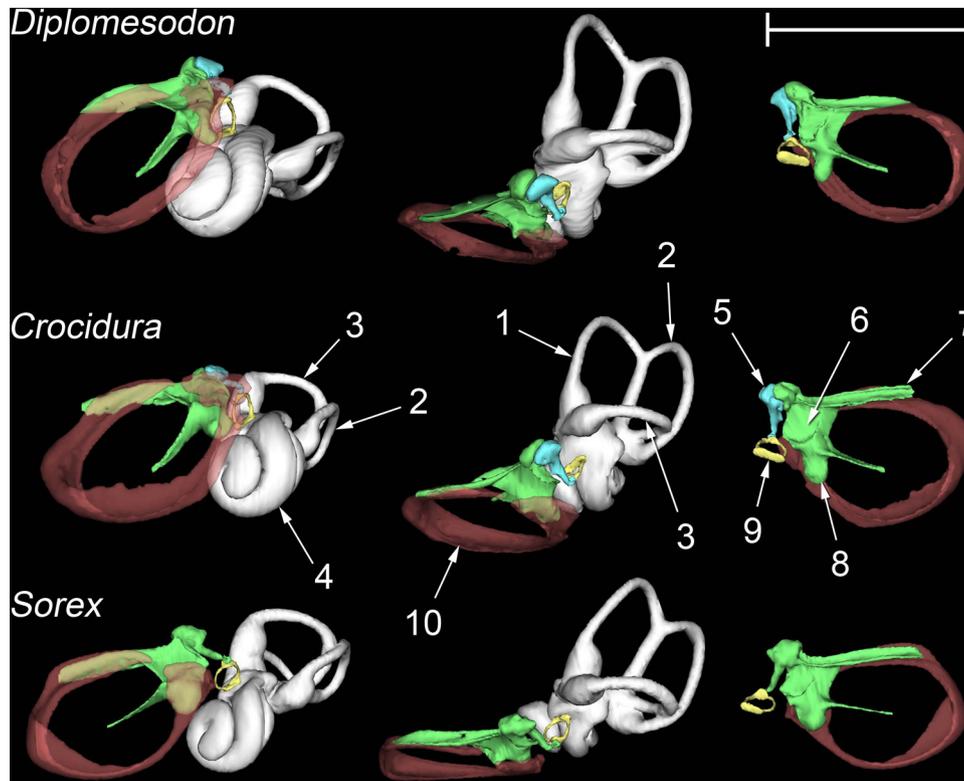


Fig. 8. Reconstructions of left middle and inner ear structures of three species of shrews: *Diplomesodon pulchellum* (subadult female; upper row), *Crocidura suaveolens* (middle row) and *Sorex araneus* (bottom row). On the left are ventral views of the middle ear ossicles, ectotympanic and bony labyrinth of each species; rostral is to the left. In the middle are lateral views of the same. On the right are the middle ear structures only, as seen from within the tympanic cavity. Scale bar 3 mm. Key: 1 = anterior semicircular canal; 2 = posterior semicircular canal; 3 = lateral semicircular canal; 4 = cochlea; 5 = incus; 6 = malleus; 7 = anterior process of malleus; 8 = orbicular apophysis of malleus; 9 = stapes; 10 = ectotympanic bone. The ectotympanic bone is shown in translucent red, the malleus in green, the stapes in yellow and the bony labyrinth in white. The incus is shown in blue except in *Sorex*, in which it is indistinguishably fused with the malleus.

represents indirect evidence of the relationship between the pulse rate of screeches and the work of body muscles. A direct relationship between pulse rate of loud trilled calls and the performance of trunk muscles (external obliques) was demonstrated using high-speed video and electromyography (EMG) *in vivo* in two related species of North American gray tree frogs (Girgenrath and Marsh, 1997). Both species produce trilled calls with high sound intensity, but the sound pulse frequency within calls in *Hyla chrysoscelis* is twice that in *Hyla versicolor*. In both species, sound pulse rate directly correlated with the active contractions of the trunk muscles. It should be noted that sound production mechanisms in frogs and mammals differ considerably, as in frogs the air is forced from the lungs into the air sac(s) with trunk muscles, which results in coincidence of the sound pulse frequency with contractions of the trunk muscles (Wells, 2007).

As the rates of screech modulations and of vibrations in piebald shrews exceed 100 Hz, the possibility that their muscles are capable of ‘superfast contractions’ should be investigated. Such contractions have been found in syringial muscles of songbirds (Elemans et al., 2008), laryngeal muscles of buzzing bats (Elemans et al., 2011; Ratcliffe et al., 2013), swimbladder muscles of some fishes (Rome et al., 1996; Rome, 2006) and tail shaker muscles of rattlesnakes (Conley and Lindstedt, 1996; Rome et al., 1996). In all cases, these superfast contracting muscles are associated with acoustic signal production.

In *Diplomesodon*, there is a consistent increase in the pulse rate of screeches during ontogeny. Two species of flat-headed bats, *Tylonycteris pachypus* and *Tylonycteris robustula* (Zhang et al., 2005), and Laxmann’s shrews, *Sorex caecutiens*, also show an increasing pulse rate of screeches with maturation. In Laxmann’s shrew pups, the pulse rate of screeches increased during postnatal development

from 78 Hz at 9 days to 173 Hz at 25 days (Movchan and Shibkov, 1983; Konstantinov and Movchan, 1985) and reached 212 Hz in adults (Movchan and Shibkov, 1982, 1983).

In this study, clicks were found to be most frequent soon after birth, and then became less frequent with age and nearly ceased by 22 days. Piebald shrews produce only audible calls below 15 kHz; we found no ultrasonic components within their vocalizations. They therefore appear not to produce the ultrasonic orientation clicks described for other species of shrews (Gould et al., 1964; Tomasi, 1979; Forsman and Malmquist, 1988; Thomas and Jalili, 2004) and for tenrecs (Gould, 1965). Clicks were most commonly made by helpless, blind newborns with closed ears, usually when placing the pups on the flat surface of a table. If pups use clicks for orientation, the low fundamental frequency of these signals might be related to the lack of a patent external auditory meatus. However, the helpless and blind pups hardly need orientation calls, as their ability to move is very limited even if they had information about their environment. A more likely explanation is that clicks are normally used for attracting attention of the mother: the f_0 of newborn clicks found in this study (9.2 kHz in 1–2 day-old pups) falls within the optimum hearing range of adult piebald shrews, which is approximately between 7 and 30 kHz (Konstantinov et al., 1987). The clicks are probably made with the tongue, as has been reported for other species of shrews (Gould, 1969). In adult piebald shrews, clicks are very rare and were documented only when the animal was placed on an elevated disk (Volodin et al., 2012, 2015b).

In piebald shrews, the f_0 of squeaks and clicks remained unchanged in spite of the age-related increase in body mass and head length; moreover, the f_0 of screeches increased with age. This pathway of f_0 ontogeny contrasts with the steady decrease of f_0 with age which is more typical of mammals, an effect of the

growth of sound-producing components of the vocal apparatus (Morton, 1977; Fitch and Hauser, 2002; Matrosova et al., 2007). A descending f₀ during postnatal development was found in primates (Inoue, 1988; Hammerschmidt et al., 2000, 2001; Pistorio et al., 2006; Ey et al., 2007), elephants (Stoeger-Horwath et al., 2007), rodents (Owings and Loughry, 1985; Nesterova, 1996; Blumstein and Munos, 2005), bovids (Briefer and McElligott, 2011; Efremova et al., 2011; Volodin et al., 2014) and cervids (Torriani et al., 2006; Volodin et al., 2015a).

An increase of f₀ of echolocation and social calls with age is known for many species of bats (Jones et al., 1991; de Faniis and Jones, 1995; Moss et al., 1997; Zhang et al., 2005; Liu et al., 2007; Hiryu and Riquimaroux, 2011; Monroy et al., 2011; Jin et al., 2011, 2012; Funakoshi et al., 2013; Wang et al., 2014). This might result from selection pressures for avoiding obstacles or spotting prey items. The higher the f₀ of the echolocation pulse, the higher the directionality of the sound wave and the higher the precision of location of the object by the reflected echo (Madsen and Surlykke, 2013). Throughout maturation, bat pups produce signals of an increasingly high fundamental frequency using the thin vocal membranes on their vocal folds (Novick and Griffin, 1961; Suthers and Fattu, 1973).

The f₀s of screams of the mothers and 1–4 month old pups of sea otters (*Enhydra lutris*) were indistinguishable (McShane et al., 1995). That study was devoted to description of the vocal repertoire and individual traits in calls of sea otters, and the indistinguishable f₀s between pups and adults were not discussed by the authors. In contrast, in the Asian small-clawed otter *Aonyx cinerea*, the f₀ in contact calls was lower in adults compared to adolescents (Lemasson et al., 2014).

Among tonal calls of Asian house shrews, only twitters and chirps were found in both pups and adults. In twitters, the f₀ is halved from pups to adults, whereas the f₀ of chirps remains unchanged throughout ontogeny (Schneiderová, 2014). For five species of ground squirrels (genus *Spermophilus*), the f₀s of alarm calls were indistinguishable between pups and adults (Matrosova et al., 2007, 2012; Swan and Hare, 2008; Volodina et al., 2010). Playbacks confirmed that adult Richardson's ground squirrels *S. richardsonii* did not discriminate between calls of pups and adults (Swan and Hare, 2008). It has been proposed that pups mimic calls of adults, in order to decrease the age-dependent risk of predation and infanticide (Matrosova et al., 2007; Volodina et al., 2010).

We have shown that shrews represent another group of mammals which do not show a descent in call fundamental frequency as they grow from pups to adults. The available data are insufficient to explain the reason underlying this phenomenon. It is doubtful that similar f₀s of pups and adults result from an accelerated growth of the pup larynx compared to the growth of the rest of the body. In speckled and yellow ground squirrels (*Spermophilus suslicus* and *S. fulvus*) the size of the larynx is proportional to the condylobasal length of the skull, across both pups and adults (Matrosova et al., 2007), despite the alarm call f₀s being indistinguishable. Although mammalian juveniles have relatively larger heads compared to body size than adults, their absolute head sizes differ considerably: in the piebald shrew, the newborn head length comprises only 48.6% of the adult head length (Zaytseva et al., 2013).

4.2. Vibration production and ear morphology

A previous study demonstrated the abilities of adult and subadult piebald shrews from the 34th day post-partum to produce seismic vibrations, the frequency of which was the same as the pulse rate of screeches in the same individuals (Volodin et al., 2012). Although the relationship between vibration frequency and the rate of the deep sinusoidal modulation (pulse rate) of screeches has not yet been directly confirmed for piebald shrews, the developmental

pathway of the pulse rate of screeches might point to the developmental pathway of vibration production in this species. We did not measure seismic vibrations in the present study, but the vibrating behaviour of piebald shrews is very characteristic and obvious, and this was not observed in our animals (our observations extended up to the 22nd days of their lives). Vibrating behaviour therefore must appear later in the ontogeny of piebald shrews.

The ears of *Sorex* and *Crocicidura* shrews have been fairly well described, one of the most comprehensive accounts being the paper by Burda (1979). Our anatomical findings for these genera are consistent with Burda's report. In contrast, the only information relating to the ear of *Diplomesodon* that the authors are aware of is a single illustration of the auditory region of the skull, minus ossicles, in McDowell (1958). We found the ear structures of *Diplomesodon* to be very similar to those of *Crocicidura* and *Sorex*; such differences that did exist tended to separate *Sorex* from the other two. The apparent fusion of malleus and incus in *Sorex* is an unusual feature of shrews of this genus (Mason and Farr, 2013).

Diplomesodon has been found in molecular studies to emerge from within the monophyletic genus *Crocicidura* (Dubey et al., 2008). From a phylogenetic point of view, the close similarity between the ear structures of *Diplomesodon* and *C. suaveolens* is therefore unsurprising. However, the skull of *Diplomesodon* is relatively shorter and broader than that of other shrews, perhaps relating to its burrowing habits (Heptner, 1939), and *Diplomesodon* is the only shrew so far known to produce vibratory signals. There was, then, reason to suspect that the ear region of this animal might be specially adapted to reflect these behaviours, but our morphological comparison has failed to show that the *Diplomesodon* ear differs substantially from that of other shrews, at least in terms of the hard structures visible in CT scans.

Among rodents, the mole-rat *Spalax* (Spalacidae) communicates by means of seismic vibrations of a few hundred Hertz generated by "head-drumming" on the burrow roof (Heth et al., 1987; Rado et al., 1987); bathyergid mole-rats (Bathyergidae) and kangaroo rats (Heteromyidae) also communicate using seismic signals (Narins et al., 1992; Randall, 2010). Although it is not yet known for sure whether the middle ear represents the means of detection of these signals, all of these rodents have a "freely mobile" ossicular morphology, associated with low-frequency hearing (Mason, 2015). The Namib desert golden mole *Eremitalpa* is believed to be able to detect low-frequency seismic vibrations generated by grassy tussocks in the wind, and by the insect prey that live therein (Narins et al., 1997; Lewis et al., 2006). This Afrotherian insectivore has extraordinarily enlarged middle ear ossicles which appear to be suited to vibration detection through a form of inertial bone conduction (Mason, 2003a,b; Willi et al., 2006b). In contrast, the 'microtype' ear morphology of *Diplomesodon*, also found in other shrews, bats and mice, is associated with good high-frequency hearing (Fleischer, 1978; Mason, 2013). Consistent with this, evoked potentials in response to airborne sound were found in *Diplomesodon* and several other shrew species at frequencies from around 1 to 70 kHz (Konstantinov et al., 1987). The ear morphology of the piebald shrew is therefore quite unlike the ear morphology of mammalian species which are known to make use of seismic vibrations, and it shows no clear signs of being tuned to the low seismic frequencies that this shrew produces.

Although it is possible that the ears of *Diplomesodon* in particular and perhaps shrews in general have some alternative and as-yet unidentified means of augmenting seismic sensitivity, there is currently no evidence for this. Somatosensation has been implicated in the detection of seismic cues in certain subterranean mammals which lack enlarged middle ear ossicles (see Mason and Narins, 2010; for a review), and it may well be that seismic sensitivity in *Diplomesodon* is based on this sensory modality. Although controversial (see Rado et al., 1998), some believe that *Spalax* uses a

somatosensory rather than an auditory route for seismic detection (Nevo et al., 1991). The lamellated corpuscles found in the skin of the paws may subserve this sensory modality when the animal is performing 'seismic echolocation' (Kimchi et al., 2005) and similar receptors have also been found in the skin of the rhinarium of this species (Klauer et al., 1997). Piebald shrews have ridges of fine hairs on their feet (Heptner, 1939), which start growing from 7 days of age (Vakhrusheva and Ilchenko, 1995, 2010) and are well-developed at 34 days, when vibratory behaviour has been recorded (Volodin et al., 2012). It is possible that such hairs might mediate vibrational sensitivity in the shrew, the auditory route being used mainly for perception of higher-frequency, vocal cues. However, ridges of hairs on the feet are quite common in desert species and they may help *Diplomesodon* to walk on the sand (Heptner, 1939): they are not necessarily related to seismic sensitivity.

It is interesting to speculate that the vibratory behaviour of *Diplomesodon* might relate to its semidesert habitat, an unusual environment for a shrew. Sand is regarded as a favourable medium for the propagation and localization of biological signals (Brownell, 1977); many desert animals from diverse groups including the scorpions studied by Brownell, desert ants (Buehlmann et al., 2012), the sandfish lizard (Hetherington, 1992) and the desert golden mole discussed above appear to be able to detect and make use of vibrations in sand. While it remains to be proven that *Diplomesodon* can detect the vibrations that it generates, which might potentially be used for prey detection, orientation or intraspecific communication, it would be surprising if it could not. A histological examination of the feet of this desert shrew is required to look for the presence of lamellated receptor organs, which might subserve vibratory sensitivity.

While many studies have related vertebrate seismic sensitivity to ear morphology, far fewer have considered the structural relations between the spectra of seismic and acoustic signals (Volodin et al., 2012, 2015b, Bednářová et al., 2013). Bednářová et al., (2013) discuss the possible structural and functional relationships between seismic and acoustic components of mechanically produced sounds in giant mole-rats (*Fukomys mechowii*) in relation to differential propagation of these two components in the environment, which might underlie different communication functions. However, these proposals have not yet been tested experimentally. In the case of the piebald shrew, high-frequency acoustic cues may be perceived in the normal way by the apparently unmodified auditory system, while seismic vibrations could potentially be detected using the somatosensory system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.beproc.2015.06.012>

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