Postnatal hearing development limits early parent-to-offspring vocal communication and gates sensorimotor learning in the zebra finch

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ABSTRACT

Hearing is critical for vocal communication, and in birds its onset is linked to egg hatching. Hearing develops after hatching in early hatching (altricial) species, while already functional in the egg in later hatching (precocial) species. Recent work suggests that altricial zebra finches exhibit parent-to-embryo acoustic communication, requiring functional, high-frequency hearing already in the egg. Yet, the early postnatal development of their auditory sensitivity remains unknown. By measuring hearing and vibration-detection capacity during postnatal development in zebra finches, we show that zebra finch hatchlings are deaf even to loud, broadband sounds. Auditory nerve sensitivity develops gradually in a low-to-high frequency manner. Auditory nerve responses show adult-like properties only after 20 days from hatching. Our data provide evidence against the plausibility of early parent-to-offspring acoustic communication in zebra finches and suggest that hearing functionality may set the onset of vocal learning in zebra finches.

INTRODUCTION

The ability to detect sound is critical for vocal communication, vocal learning, and maintenance of learned vocalizations (Konishi, 1965; Marler *et al.*, 1972; Nordeen and Nordeen, 1992; Heaton, Dooling and Farabaugh, 1999; Zevin, Seidenberg and Bottjer, 2004). In terrestrial vertebrates hearing improves during development through growth and maturation of the inner and middle ear. In birds, the onset of hearing capacity is often linked to the timing of egg hatching, with many precocial bird species hatching later than altricial birds. For example, the average incubation time of precocial chickens and ducks is 21 and 28 days while for altricial zebra finches and budgerigars it is 14 and 18 days, respectively (Hamburger and Hamilton, 1951; Montgomery, Burke and Byers, 1978; Ar and Tazawa, 1999; Murray *et al.*, 2013).

The hearing of altricial birds is widely believed to mature after hatching with little to no functional hearing during the embryonic, pre-hatching stages (Woolley, 2017). In contrast, precocial bird species show auditory function already while still being inside the egg (Gos, 1933; Gottlieb, 1965; Konishi, 1973; Saunders, Coles and Gates, 1973). The embryonic onset of hearing function in precocial birds enables embryo-to-embryo (Vince, 1966; Woolf, Bixby and Capranica, 1976; Schwagmeyer *et al.*, 1991; Noguera and Velando, 2019), and parent-to-embryo vibroacoustic communication where it plays a crucial role in imprinting on species-specific sounds (Grier, Counter and Shearer, 1967; Hess, 1972; Gottlieb, 1979). Embryonic sensitivity to vibroacoustic cues mediates synchronization and induction of hatching in other precocial animals, including frogs (Warkentin, Caldwell and McDaniel, 2006), snakes (Aubret *et al.*, 2016), turtles (Doody *et al.*, 2012; McGlashan *et al.*, 2015), and crocodiles (Vergne and Mathevon, 2008).

Surprisingly, recent research suggests that parent-to-embryo vibroacoustic communication may also occur in altricial birds (Colombelli-Négrel *et al.*, 2012, 2016; Colombelli-Négrel, Hauber and Kleindorfer, 2014; Kleindorfer *et al.*, 2024). This challenges the previous distinction between precocial and altricial birds in terms of functional embryonic hearing. In additional support of a less rigid distinction, a recent series of studies suggest that altricial zebra finch embryos can detect sound while still being inside the egg. When in a hot environment, zebra finch parents emit “heat calls” with a fundamental frequency of 7-10 kHz that have been proposed to epigenetically guide their unhatched offspring to adapt to high

temperatures (Mariette and Buchanan, 2016, 2019; Katsis *et al.*, 2018, 2021, 2023; Mariette *et al.*, 2018; Pessato *et al.*, 2020; Udino *et al.*, 2021; Udino and Mariette, 2022). The presence of such parent-to-embryo communication via the auditory modality crucially requires that zebra finch embryos can detect these sound stimuli in the egg. Furthermore, under the assumption that no sudden loss of hearing function occurs around hatching, these studies also imply that zebra finches must be able to hear upon hatching.

The above predictions contradict the prevailing understanding that zebra finch hearing develops predominantly after hatching. The earliest measurements of hearing sensitivity in zebra finches are at 10 days-post-hatch (DPH) and indicate a reduced hearing sensitivity of 25 dB sound pressure level (SPL) compared to 20 DPH when their hearing sensitivity is comparable to adult levels (Amin, Doupe and Theunissen, 2007). The adult frequency sensitivity ranges from 0.25-to-8 kHz with the highest sensitivity around 3 kHz (Okanoya and Dooling, 1987; Amin, Doupe and Theunissen, 2007; Yeh, Rivera and Woolley, 2023). If embryonic detection of heat calls relies on the auditory system, either through air-borne sound pressure waves on the tympanum or via bone conduction (Sakabe, Arayama and Suzuki, 1969; Mudry and Tjellström, 2011), then the onset of zebra finch hearing must occur earlier than currently assumed. As an alternative route of detection, heat call acoustic signals could induce egg vibrations that embryos sense as vibrotactile signals. However, both hearing capacity via air or bone conduction remain unknown during the early development in zebra finches. To test the hypothesis that zebra finches have functional hearing prior to hatching and possibly even before, it is imperative to conduct measurements of hearing sensitivity at early postnatal ages.

Hearing plays a crucial role in another key behaviour in songbirds, vocal learning which is the ability to learn song by imitation. Juvenile male zebra finches listen to the song of adult tutors and form a memory template of it during a restricted sensory learning phase (Immelmann, 1967). This phase is followed by a sensorimotor phase, where the birds practice their own vocalizations through trial-and-error learning until they match their internal song template. In the zebra finch, the dominant model for studying neural circuitry and behaviour underlying this complex motor learning process, the sensory and sensorimotor phase partly overlap and start on 25 DPH (Immelmann, 1967; Böhner, 1990; Roper and Zann, 2006; Braaten, 2010; Gobes, Jennings and Maeda, 2019). Experimentally priming early sensorimotor learning can push the

sensory phase to start around 20 DPH (Leitão and Gahr, 2024). The onset of the sensory phase thus closely coincides with hearing sensitivity maturation, which raises the question if hearing maturation constrains the onset of sensory and sensorimotor learning in zebra finches. However, our current understanding is limited by the lack of data on the maturation of hearing in songbirds (Woolley, 2017).

Here we test the hypothesis that zebra fiches possess auditory function at early post-hatch ages, by measuring air conduction- (AC-) and bone conduction-induced (BC-) auditory brainstem responses (ABRs). ABR recordings allow hearing threshold estimation when behavioural training is challenging (Heffner, Koay and Heffner, 2008). Additionally, we measured the vibrations that air conducted sounds can induce on zebra finch eggs. We show that the earliest AC-ABRs evoked by intense, broadband sounds could not be detected on 2 DPH, but earliest at 4 DPH. Responses to frequency specific sounds develop later and gradually in a low-to-high frequency manner. AC-ABR thresholds remain high throughout development for frequencies associated with heat calls. AC-ABR sensitivity for clicks matures to adult-like levels at 10 DPH, but the response amplitude continues to increase beyond 20 DPH. Head vibrations surprisingly do not elicit BC-ABRs in zebra finches. Airborne sounds induce low amplitude vibrations in eggs that are far below avian or mammalian vibrotactile detection thresholds. Together, our findings provide evidence against the hypothesis that zebra finch embryos and early hatchlings can detect vibroacoustic stimuli via air and bone conduction hearing or via vibrotactile senses. Our results also suggest that neural responses to sounds at the level of the auditory nerve continue to mature past 20 DPH, which suggests that hearing functionality may set the onset of vocal learning in zebra finches.

RESULTS

AC-ABRs to loud clicks appear during the first postnatal week

To assess the development of zebra finch hearing function we quantified the responses of auditory pathway neurons to auditory stimuli by recording AC-ABRs (Fig. 1a) throughout postnatal development. First, to evaluate overall hearing sensitivity we presented loud yet physiologically relevant (95 dB SPL) clicks that stimulate the auditory system across a wide frequency spectrum (Fig. 1b). Clicks activate most auditory neurons nearly simultaneously and result in prominent AC-ABRs (Jones, Jones and Shukla, 1997), thereby providing maximal sensitivity for threshold detection.

None of the tested animals displayed click-evoked AC-ABRs at 2 DPH (both with detection at signal-to-noise ratio of 2 (S/N > 2) and with detailed visual inspection; n=0/15; Fig. 1c,d). However, during the first postnatal week, click-evoked AC-ABRs became observable in some animals (S/N > 2; 4 DPH, n = 5/13; 6 DPH, n = 9/10; Fig. 1d). Subsequently, from the second postnatal week onwards, all tested animals consistently demonstrated click-evoked AC-ABRs (S/N > 2; 8 DPH, n = 11; 10 DPH, n = 11; 20 DPH, n = 7; 25 DPH, n = 8; Adult, n = 8; Fig. 1d). Thus, sounds at loud yet physiologically relevant SPLs do not evoke AC-ABRs in young zebra finches in the days after hatching.

Detection of click-evoked AC-ABRs is adult-like at 10 DPH

To estimate thresholds of hearing over postnatal age, we recorded series of click-evoked AC-ABRs at 5 dB downward steps from 95 dB SPL (Fig. 1e). Hearing thresholds are strongly affected by the age of the animal ($F(6)=25.22$, $p=4.93^{-14}$; Fig. 1f, Supplementary Table 1). Click-evoked AC-ABR thresholds are significantly higher than adult-like levels (40.6 ± 4.0 dB SPL) in animals younger than 10 DPH, but not in older animals (4 DPH vs adult, $t = 8.16$, $p = 3.27*10^{-10}$; 6 DPH vs adult, $t = 5.91$; $p = 1.38*10^{-6}$; DPH 8 vs adult, $t = 3.37$, $p = 0.008$; 10 DPH vs adult, $t = 1.23$, $p = 1$; 20 DPH vs adult, $t = -0.13$, $p = 1$; 25 DPH vs adult, $t = -1.29$, $p = 1$; p-values are Bonferroni-corrected; Fig. 1f, Supplementary Table 1). Click thresholds are as high as 80.6 ± 1.6 dB SPL on 4 DPH. Therefore, zebra finch hearing sensitivity rapidly increases during the first two postnatal weeks.

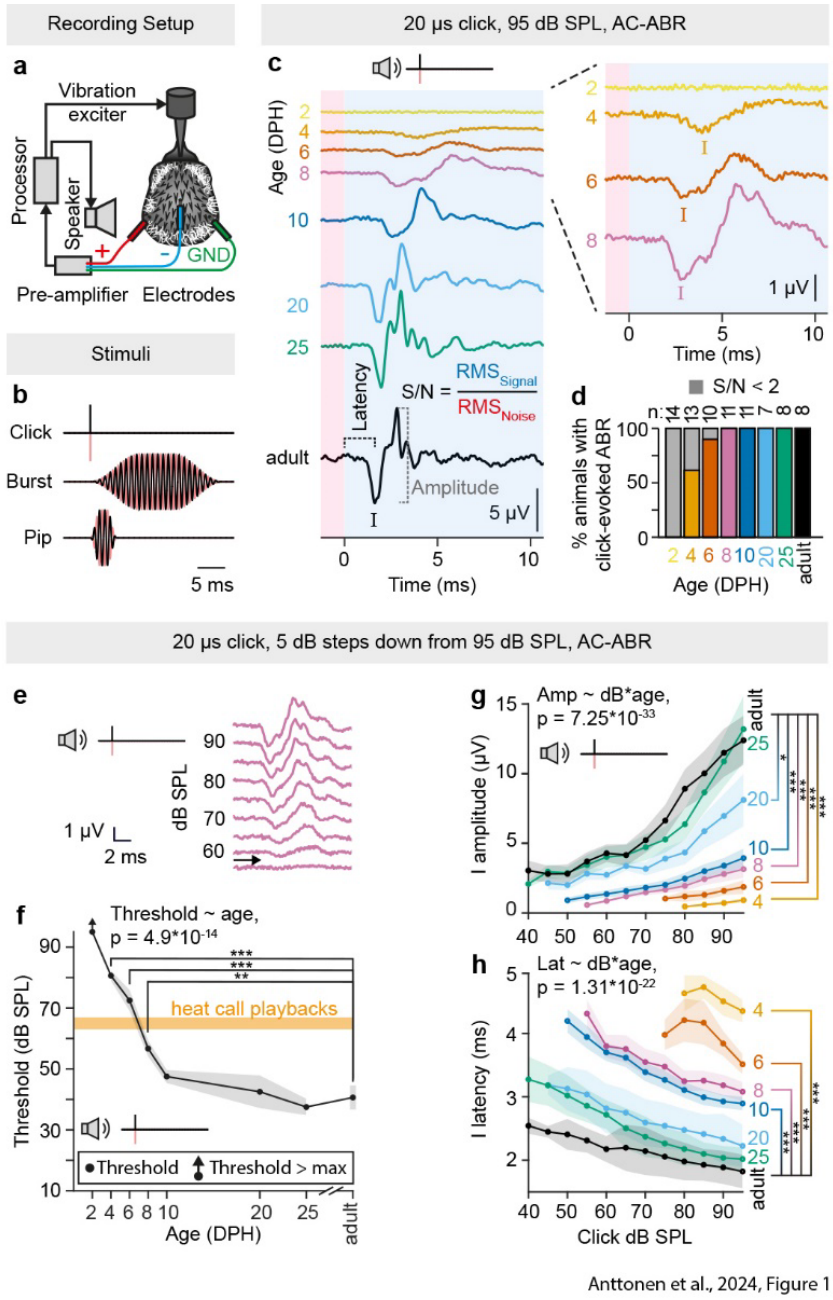


Figure 1. Click-evoked AC-ABRs appear and subsequently mature after hatching in zebra finches. a ABR recording setup. **b** Used sound stimuli. *Red*, phase inversed signal. **c** Representative AC-ABRs to 95 dB SPL clicks at tested DPH timepoints. The first negative peak (*I*) and the following trough are designated as wave I. Noise RMS is determined from the *red area* and signal RMS from the *blue area*. **d** Percentage of animals that exhibited a detectable (S/N > 2) click-induced AC-ABR. **e** Detection thresholds were detected with a set of clicks with decreasing SPL (5 dB steps). *Arrow* indicates the determined threshold for this 8 DPH example. **f** Average click thresholds at different DPH ages. Orange box indicates heat call playback levels used by Katsis et al., (2018). **g** Click-evoked AC-ABR wave I amplitude and **h** latency over SPL. Age groups are color coded in c,d,e,g,h. *Gray areas* in f-h = s.e.m.

AC-ABR waveforms change past the sensory learning onset

The click-evoked AC-ABR consists of multiple waves that reflect the subsequent activations of various neural populations of the auditory pathway. The first wave (wave I in Fig. 1c) corresponds to the activity of the primary auditory neurons (Buchwald and Huang, 1975). To study the postnatal maturation of this synchronized neural output, we measured changes in both amplitude and latency of wave I across developmental age.

In adults, the amplitude of wave I increases with the SPL of the click (Fig. 1c,g) as it has been reported for humans, mice, and budgerigars (Hecox and Galambos, 1974; Henry and Haythorn, 1978; Brittan-Powell and Dooling, 2004). We found that the age of the animal significantly affects both wave I amplitude directly and the relation between SPL and wave I amplitude (Age-SPL interaction compared to only additive effects of age and SPL: $\chi^2(6)=164.23$, $p=7.52*10^{-33}$, additive effects of age and SPL compared to only SPL: $\chi^2(6)=79.43$, $p=4.69*10^{-15}$, SPL only compared to null model: $\chi^2(1)=438.12$, $p=2.78*10^{-97}$, Fig. 1g, Supplementary Table 1). The steepness of the amplitude-SPL slope is significantly different between adults and younger animals but not between adult and those aged 25 DPH (4 DPH vs adult, $t = -5.40$, $p = 1.50*10^{-6}$; 6 DPH vs adult, $t = -5.65$, $p = 1.8910^{-6}$; DPH 8 vs adult, $t = -6.30$, $p = 3.79*10^{-7}$; 10 DPH vs adult, $t = -5.53$, $p = 6.53*10^{-6}$; 20 DPH vs adult, $t = -3.00$, $p = 0.03$; 25 DPH vs adult, $t = -0.69$, $p = 1$; p-values are Bonferroni-corrected; Fig.- 1g, Supplementary Table 1).

The latency of wave I decreases with increasing click SPL (Fig. 1c,h) consistent with findings in humans, mice, and budgerigars (Hecox and Galambos, 1974; Henry and Haythorn, 1978; Brittan-Powell and Dooling, 2004). Similar to amplitude, the age of the animal significantly affects the relation between dB SPL and wave I latency (Age-SPL interaction compared to only additive effects of age and SPL: $\chi^2(6) = 116$, $p = 1.31*10^{-22}$; additive effects of age and SPL compared to only SPL, $\chi^2(6) = 109$, $p = 2.76*10^{-21}$; SPL only compared to null model: $\chi^2(1) = 754$, $p = 4.81*10^{-166}$; Fig. 1h, Supplementary Table 1). The observed latencies are significantly longer during early postnatal development (4-to-10 DPH) when compared to latencies observed in adults. No significant differences are observed between the latencies of adults and animals older than 10 DPH (4 DPH vs adult, $t = 12.56$, $p = 3.41*10^{-19}$; 6 DPH vs adult, $t = 10.06$, $p = 1.49*10^{-13}$; 8 DPH vs adult, $t =7.24$, $p = 1.16*10^{-8}$; 10 DPH vs adult, $t = 6.18$, $p = 5.95*10^{-7}$; 20 DPH vs adult, $t = 2.23$, $p = 0.18$; 25 DPH vs adult, $t = 1.67$, $p = 0.61$; p-values are Bonferroni-corrected; Fig. 1h, Supplementary Table 1).

The observed changes in the synchronized output of the auditory nerve indicate that peripheral encoding of sounds in zebra finches mature during the first three weeks post hatching and resemble the responses seen in adults at 25 DPH.

Hearing sensitivity to high frequency sounds develops last

While clicks provide general sensitivity thresholds, they do not inform about frequency sensitivity of the auditory system. To study frequency-specific hearing sensitivity we tested whether loud (95 dB SPL) tone bursts centred at specific frequencies between 0.25-to-8 kHz elicit detectable AC-ABRs. Each stimulus was 25 ms in length so that the lowest studied frequency stimulus would undergo at least 6 cycles during this period. The rise/fall time of the stimulus was set to 5 ms to minimize spectral splatter that compromises the frequency specificity of the stimulus (Lauridsen, Brandt and Christensen-Dalsgaard, 2021).

Tone burst-evoked AC-ABRs are detectable for the first time at 8 DPH at 1 kHz (Fig. 2a and Supplementary Figure 1a). As observed with click stimuli, the amplitude of the tone burst-induced AC-ABRs appears to increase (Fig. 2a and Supplemental Fig. 1b; Age as a predictor of amplitude compared to null model: $\chi^2(3) = 6.11$, $p = 0.11$, Supplementary Table 2) while the latency significantly decreases during postnatal development (Fig. 2a and Supplementary Figure 1c; Age as a predictor of latency compared to null model: $\chi^2(3) = 33.72$, $p = 2.27*10^{-7}$, Supplementary Table 2). Latencies are significantly longer at 8-to-10 DPH (Latencies at 1 kHz: 8 DPH, 6.60 ± 0.77 ms; 10 DPH, 5.01 ± 0.40 ms) and are adult-like at 20-to-25 DPH (Latencies at 1 kHz: 20-to-25 DPH, 3.34 ± 0.18 ms; adult, 3.67± 0.30 ms; 8 DPH vs adult, $t = 5.95$, $p = 1.87*10^{-6}$; 10 DPH vs adult, $t = 4.52$, $p = 0.0004$; 20-to-25 DPH vs adult, $t = 1.16$, $p = 0.81$; p-values are Bonferroni-corrected; Supplementary Table 2). During the following weeks of postnatal development, tone burst-induced AC-ABRs became detectable at 0.5-to-4 kHz (Supplementary Fig. 1a). No responses were detected at higher frequencies (Fig. 2b and Supplementary Fig. 1a). Taken together, we observed that maturation of the hearing sensitivity in zebra finches progresses in a low-to-high frequency order, as is also observed in mice and cats (Ehret, 1976; Ehret and Romand, 1981) and in other birds (Brittan-Powell and Dooling, 2004; Kraemer *et al.*, 2017).

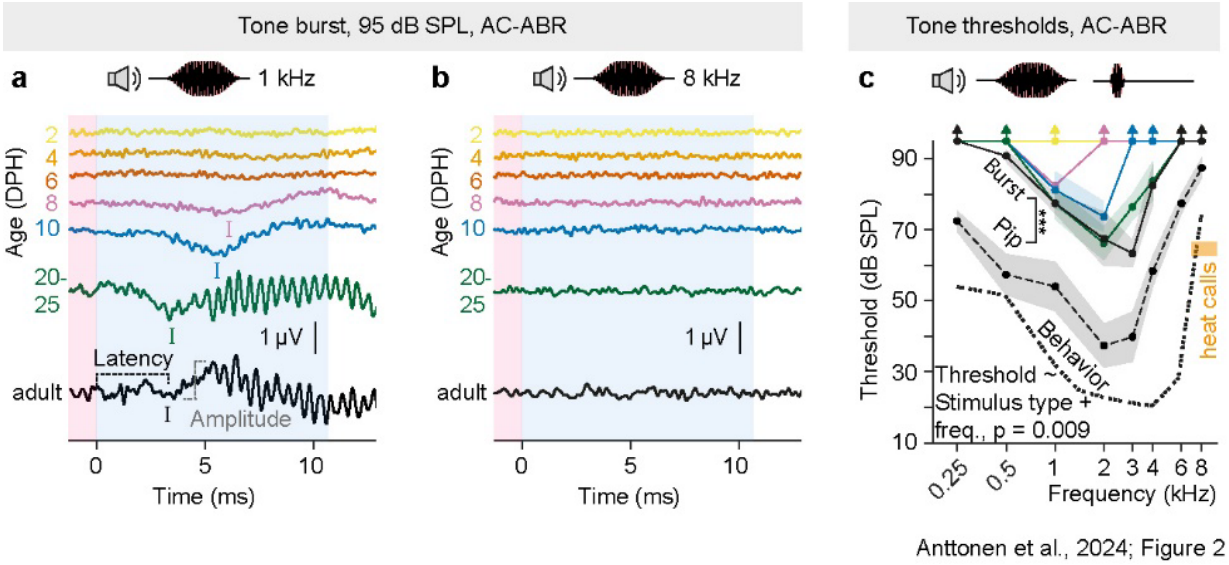


Figure 2. Frequency dependent hearing maturation over postnatal development. a Representative AC-ABRs to 1 kHz and **b** 8 kHz tone burst stimuli over postnatal development. **c** Zebra finch ABR thresholds obtained with tone bursts (*solid lines*, this study) with tone pips (*dashed line*., this study). Behavioural hearing thresholds recorded by Okanoya and Dooling (1987) are plotted with a *dotted line*. *Orange box* displays the playback level of heat calls used by Katsis et al. (2018). Shaded areas = s.e.m.

Limited hearing sensitivity at heat call frequencies

To test how readily the adult zebra finch auditory system detects heat calls, we analysed their hearing sensitivity for higher frequency sound stimuli in more detail. Importantly, tone bursts evoked AC-ABRs only in about half of the adult zebra finches (Supplementary Fig. 1a) and the detection thresholds of > 60 dB SPL were high. This indicates that while being more frequency specific, tone bursts failed to recruit zebra finch auditory neurons to fire concurrently enough to generate detectable AC-ABRs. A significant effect of age on thresholds could not be observed (Age as a predictor of threshold compared to null model: $\chi^2(3) = 1.64$, $p = 0.65$, Supplementary Table 2), likely due to the low statistical power caused by the limited amount of observed tone burst-evoked AC-ABRs.

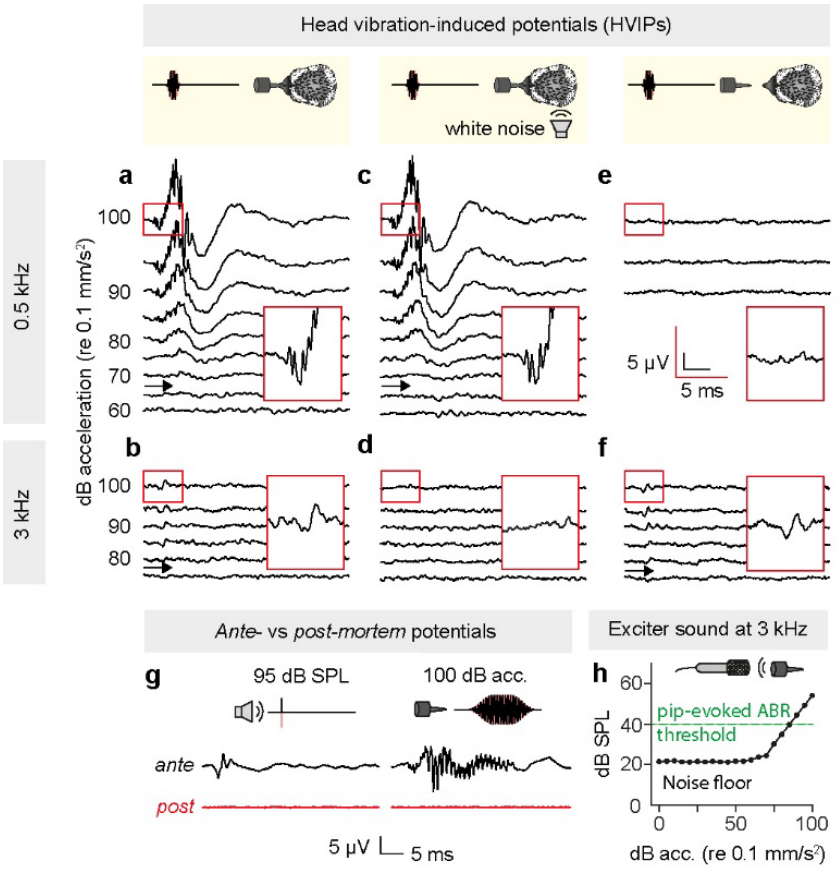
Next, we designed sound stimuli that aim to recruit more auditory neurons to fire simultaneously. We shortened the stimulus rise/fall time to 1 ms and the plateau time to 3 ms. The obtained 5 ms-long tone pips (Fig. 1b) have a broader spectral distribution of acoustic energy when compared to tone bursts (Lauridsen, Brandt and Christensen-Dalsgaard, 2021).

Compared to tone burst-induced AC-ABRs, the SPL matched tone pips produced much more prominent AC-ABRs (Fig. 2c and Supplementary Fig. 2a). Tone pip-evoked AC-ABRs were readily detected in all tested adult zebra finches around their most sensitive hearing range of 1-to-4 kHz (Okanoya and Dooling, 1987) (Supplementary Fig. 2b). Detection thresholds for tone pips are ~20 dB SPL more sensitive than those of tone bursts (Additive effects of stimulus type and frequency as predictors of threshold compared to stimulus type alone $\chi^2(1) = 6.84$, $p = 0.009$; stimulus type as a predictor of threshold compared to null model: $\chi^2(1) = 10.27$, $p = 0.001$, Supplementary Table 2). Importantly, the tone pip-based audiogram resides ~10-to-20 dB SPL above the known behavioural thresholds (Okanoya and Dooling, 1987) (Fig. 2c) as one would expect based on previous studies (Stapells, 2000). Based on these audiograms, we can conclude that heat calls at playback levels of 62-to-67 dB SPL at the ear (Katsis *et al.*, 2018) are just on or below the limit of what adult zebra finches can readily detect.

Bone conduction does not mediate hearing in zebra finches

After evaluating the development of air conduction-based hearing, we next studied whether bone conduction can mediate hearing during early postnatal development in zebra finches. By using the same electrode configuration as for AC-ABRs, we tested whether head vibrations can evoke BC-ABRs.

In adult zebra finches, tone pip- and tone burst-like head vibrations at high acceleration levels gave rise to large recordable potentials at frequencies below 2 kHz (Figs. 3a and 4a) and to smaller, ABR-like potentials at 2-to-3 kHz (Figs. 3b and 4a). Aiming first to resolve the unknown source of these responses, we tested whether a simultaneous air conduction white noise sound stimulus at 95 dB SPL masks the vibration-induced signals. Interestingly, responses below 2 kHz were not sensitive to noise masking (Fig. 3c), which strongly suggests that they did not originate from the auditory system.



Anttonen et al., 2024; Figure 3

Figure 3. Characteristics of potentials generated by head vibrations in zebra finches. *Red insets* in a-f show the start of the response at a higher magnification. **a** Tone pip vibrational stimuli induce large recordable potentials at frequencies below 2 kHz. The visualised example is at 0.5 kHz. **b** At 2-to-3 kHz, small ABR-like potentials can be recorded. This example is at 3 kHz. **c** Concurrent 95 dB SPL white noise fails to mask the large potentials but **d** eliminates the small ABR-like potentials. **e** Detaching the vibration probe from the head eliminates the large potentials while the ABR-like response remains unaffected. **g** Both response types disappear after the animal dies **h** the vibration exciter produces audible sounds at high acceleration levels.

In contrast, the head vibration-induced ABR-like responses were effectively masked by noise (Fig. 4d, n = 3) as expected for BC-ABRs. To test if head vibrations are required for generating these potentials, we lifted the vibration exciter a few millimeters to prevent direct head contact. While this adjustment eliminated the potentials with stimuli below 2 kHz (Fig. 3e), the smaller ABR-like potentials persisted (Fig. 3f, n = 3). Furthermore, the observed potentials require the animal to be alive to occur, indicating that they originate from the animal and are not artefacts of e.g. electrode vibration (Fig. 3g; n = 3). Taken together, we conclude that the ABR-like

responses at frequencies above 2 kHz are driven by something else than head vibrations, while responses below 2 kHz are driven by head vibrations.

While searching for the origin of noise-sensitive responses described above, we noticed that the vibration exciter used by us and others (Chhan, McKinnon and Rosowski, 2017) produces audible sounds in addition to vibration at high stimulus intensities. When driven for example at 3 kHz and above 85 dB re 0.1 mm s^{-2}, the exciter produces sounds above 40 dB SPL at the animals' ear that are of sufficient amplitude to evoke AC-ABRs (Fig. 3h). Therefore, we suggest that the observed ABR-like responses during vibration excitation are AC-ABRs evoked by exciter-generated sounds and not head vibration-evoked BC-ABRs.

Responses to head vibrations mature postnatally and require high acceleration

To study the postnatal development of head vibration-induced potentials, we induced head vibrations with tone bursts in zebra finches at frequencies between 0.25-to-8 kHz. We used tone bursts as they have a lower capability to induce AC-ABRs than tone pips, minimizing the AC-ABRs evoked by the audible sounds originating from the exciter. Potentials induced by head vibrations can be detected only with stimuli below 2 kHz (Fig. 4a,c). These responses typically consist of two negative deflections (n_I and n_{II}) and of a single positive one between them (p_I). The response waveform changed during postnatal development (Fig. 4b) with the number of animals displaying these responses increasing as they matured (Fig. 4c). Such responses are detected with 0.25 kHz stimulation as early as 2 DPH, followed with 0.5 kHz at 6 DPH, and finally at 1 kHz in three-week-old animals (Fig. 4c). A significant effect of age on thresholds could be observed (Age as a predictor of threshold compared to null model: $\chi^2(6) = 23.94$, $p = 0.0005$, Supplementary Table 3). The acceleration threshold levels reduce over postnatal development (2 DPH vs adult, $t = 2.74$, $p = 0.051$; 4 DPH vs adult, $t = 3.60$, $p = 0.005$; 6 DPH vs adult, $t = 2.74$, $p = 0.051$; 8 DPH vs adult, $t = 4.13$, $p = 0.003$; 10 DPH vs adult, $t = 3.68$, $p = 0.006$; 20-to-25 DPH vs adult, $t = 3.73$, $p = 0.012$; p-values are Bonferroni-corrected; Supplementary Table 3) and are around 80 dB re 0.1 mm s^{-2} in adults (Fig. 4d). Taken together, head vibration-induced potentials mature during postnatal development in a low-to-high frequency manner.

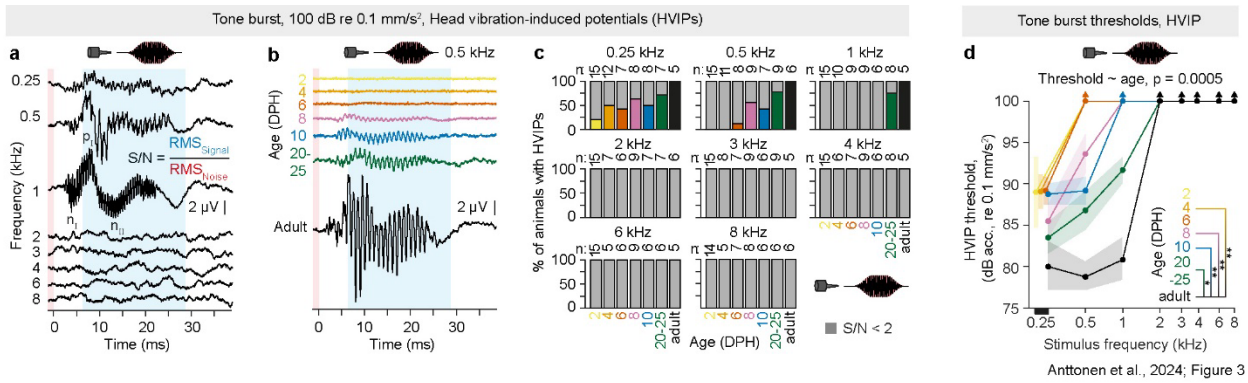
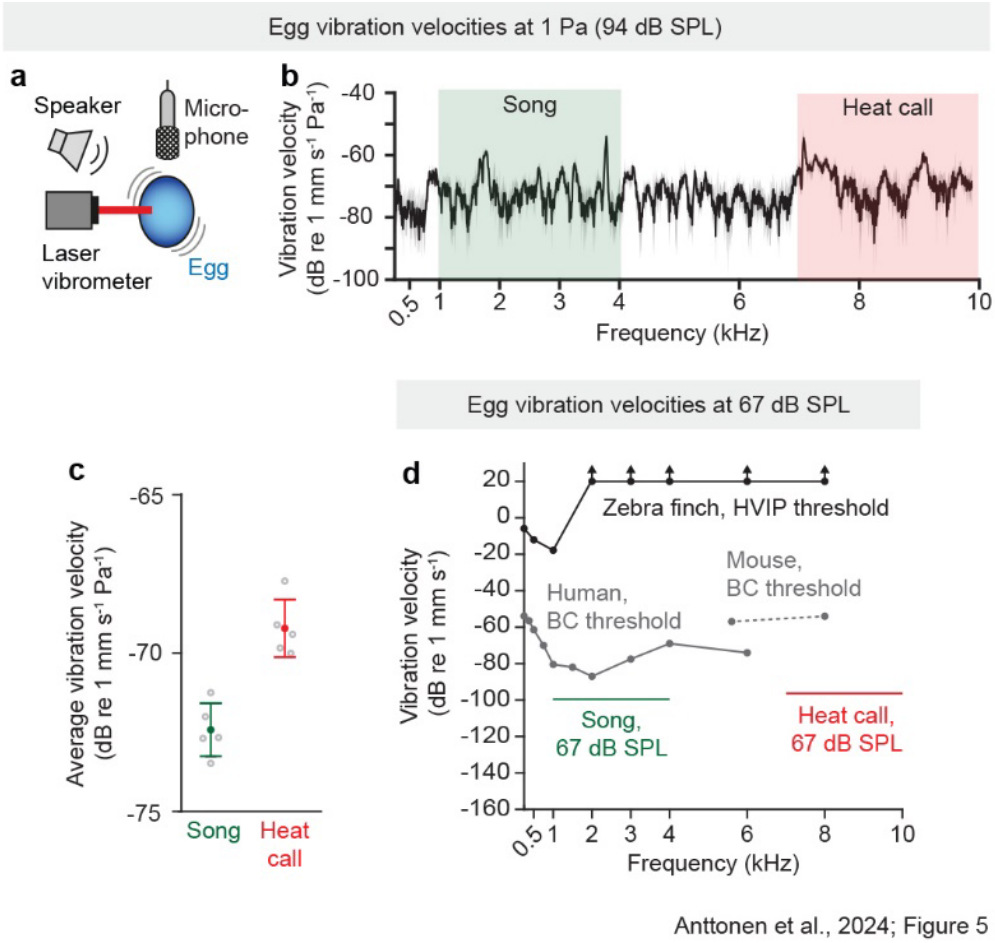


Figure 4. Postnatal maturation of head vibration-induced potentials (HVIPs). a-c relate to tone burst vibration stimuli delivered on the head at 100 dB re 0.1 mm/s^2 **a** Representative, adult HVIPs evoked by intense 0.25-to-8 kHz tone bursts. **b** Representative HVIPs induced by intense 0.5 kHz tone bursts at various DPH timepoints. **c** Percentage of animals displaying detectable HVIPs (S/N > 2) at various ages (DPH). Number of tested animals is displayed above the bars. *Gray bars* indicate animals without signal. **d** Acceleration thresholds for HVIPs at various DPH timepoints. Shaded area = s.e.m.

Sound-induced egg vibrations are below detectable levels

Our results do not support the hypothesis that embryonic zebra finches can use their immature auditory system to detect high frequency heat calls. We therefore tested the hypothesis that airborne sounds can induce zebra finch eggs to vibrate at high enough velocities to be detected via vibrotactile senses (Mountcastle, LaMotte and Carli, 1972; Hörster, 1990). We played sound frequency sweeps to zebra finch eggs while simultaneously recording the sound-induced vibrations of the egg with a laser vibrometer (Fig. 5a). The vibration velocity transfer function shows that zebra finch eggs vibrate at velocities around -85 to -55 dB re 1 mm s^{-1} when exposed to a 94 dB SPL frequency sweep between 0.25-to-10 kHz (Fig. 5b). Within the frequency ranges associated with most sensitive hearing of zebra finches (1-to-4 kHz, *green*) and with heat calls (7-to-10 kHz, *red*), sound exposures at 94 dB SPL induce average vibration velocities of -72.4 ± 0.8 and -69.2 ± 0.9 dB re 1 mm s^{-1}, respectively (standard deviation, Fig. 5c). We calculated the corresponding vibration velocity levels induced by SPLs used during experimental heat call playbacks to zebra finch eggs (67 dB SPL) (Katsis *et al.*, 2018) to be between -99.4 and -96.2 dB re 1 mm s^{-1}, respectively (Fig. 5d). The obtained vibration velocities are approximately 10-to-40 dB lower than typical vertebrate bone conduction thresholds (Håkansson, Tjellström and Rosenhall, 1985; Sakai, Karino and Kaga, 2006; Chhan, McKinnon and Rosowski, 2017), 80 dB

lower than what human (Mountcastle, LaMotte and Carli, 1972) and bird (Hörster, 1990) vibrotactile senses typically can detect, and 80 dB lower than the threshold levels to generate HVIPs in zebra finches (Fig. 5d). In conclusion, air-borne sounds at physiologically relevant SPLs are not able to induce zebra finch eggs to vibrate at high enough velocities to be detected by avian (or mammalian) vibrotactile senses or by bone conduction-mediated hearing.



Anttonen et al., 2024; Figure 5

Figure 5. Intense sound stimulation induces zebra finch eggs to vibrate at very low velocities. a Setup used to measure sound induced vibrations of eggs. **b** Average (N=5) vibration velocity transfer function of a zebra finch egg during a 94 dB SPL sound sweep between 0.25-to-10 kHz. *Gray area* marks the standard deviation. *Green* and *red areas* display the frequency windows related to the best sensitivity of zebra finch hearing (*song*) and to heat calls, respectively. **c** The average egg vibration velocities induced by a 94 dB SPL sounds at frequencies associated with song (*green*) and with heat calls (*red*). Error bars = s.d. **d** The average egg vibration velocities caused by a 67 dB SPL sounds plotted together with thresholds for human BC-ABR (Håkansson, Tjellström and Rosenhall, 1985), mouse BC-ABR (Chhan, McKinnon and Rosowski, 2017) and zebra finch HVIPs.

DISCUSSION

Our data challenge previous findings that zebra finch parents can use sound to communicate with their hatchlings and embryos through calls with high fundamental frequencies of 7-to-10 kHz. The lack of ABRs even to loud stimuli show that zebra finch hatchlings are deaf right after hatching (Fig. 1). Furthermore, the progressively increasing hearing sensitivity over postnatal development (Fig. 1) strongly suggests that zebra finch embryos should have even lower hearing sensitivity than hatchlings. Even in precocial bird species that show auditory function already during embryonic development, auditory thresholds remain very high until a few days before hatching (Saunders, Coles and Gates, 1973; Saunders, Gates and Coles, 1974; Rebillard and Rubel, 1981; Lippe, 1994). For example, evoked responses to very loud sounds (>100 dB SPL) have been obtained at around E12 in precocial chickens (21 embryonic days in total in chickens) (Saunders, Coles and Gates, 1973). However, ambient sound levels evoke responses much later around E16 (Jones, Jones and Paggett, 2006). Importantly, the above discussed measurements of embryonic hearing are often overestimations of the natural hearing sensitivity since they involve opening the egg and clearing the auditory meatus from fluids, and even drainage of fluids from the middle ear (Rebillard and Rubel, 1981). Future experiments with non-invasive techniques are required to estimate embryonic hearing capabilities of zebra finch embryos.

Our data demonstrates that hearing functionality in zebra finches gradually increases over postnatal development. The gradual increase of ABR sensitivity over postnatal development is consistent with previous observations in zebra finches, and another songbird, the pied flycatcher (Aleksandrov and Dmitrieva, 1992) as well as in other altricial bird species such as budgerigars (Brittan-Powell and Dooling, 2004), and barn owls (Köppl and Nickel, 2007; Kraemer *et al.*, 2017). It is important to note that ABR recordings are minimally invasive but crude measurements of hearing sensitivity and do not inform about the underlying processes that are behind the observed development of hearing sensitivity. These processes have been studied well in chickens. For example, the onset of evoked responses to very loud sounds coincides with synapse formation on hair cells of the chicken inner ear (Saunders, Coles and Gates, 1973; Cohen and Fermin, 1978; Caus Capdevila, Sienknecht and Köppl, 2021). The fluid unloading and maturation of the middle ear structures have been suggested to cause some of the significant improvements in auditory sensitivity during chicken development (Saunders, Coles and Gates,

1973; Cohen, Hernandez and Saunders, 1992).When these hallmark events occur in zebra finch development remains unknown. Future work on the development of the outer, middle, and inner ear of the zebra finch is thus needed to offer insights into the physiological basis for the observed postnatal improvement of hearing sensitivity.

During early postnatal development prior to 10 DPH, the hearing sensitivity of zebra finches is limited to frequencies around 1 kHz, and sensitivity for higher frequencies develops progressively afterwards. The progressive development of high frequency hearing is consistent with observations on other altricial bird species, such as budgerigars (Brittan-Powell and Dooling, 2004) and precocial bird species such as chickens, ducks, and geese (Konishi, 1973; Saunders, Coles and Gates, 1973; Saunders, Gates and Coles, 1974; Rebillard and Rubel, 1981; Cohen, Hernandez and Saunders, 1992; Dmitrieva and Gottlieb, 1992). Therefore, the functional maturation of hearing in zebra finches follows a gradual, progressive pattern consistent with other bird species. The late maturation of high frequency hearing sensitivity combined with the fact that heat calls consist of high fundamental frequencies (7-to-10 kHz) make heat calls particularly challenging to be detected by developing zebra finches - even so by adults (Fig. 2). This difficulty in detection is not unique to zebra finches; even precocial species that display hearing function before hatching have high response thresholds for sounds above 6 kHz as neonates (chicken: >120 dB SPL; mallard duck: >90 dB SPL) (Saunders, Coles and Gates, 1973; Dmitrieva and Gottlieb, 1992). Consistent with limited high frequency hearing, the established parent-embryo communication of precocial mallard ducks depends specifically on the low-frequency auditory sensitivity of the embryo to detect maternal calls with frequency components below 3 kHz (Gottlieb, 1975). Taken together, our results provide evidence against the hypothesis that zebra finch embryos can hear heat calls, and we consider it thus unlikely that adult zebra finches can communicate with their embryos via air-borne sound.

Sound-induced vibrations of the egg and bone conduction hearing could provide an alternative explanation for the detection of heat calls by zebra finch embryos (Mariette, 2024). However, the apparent lack of BC-ABRs suggest that bone conduction hearing experienced by zebra finches is limited or non-existent (Figs 3, 4). This finding is surprising as bone conduction hearing functions in multiple other species (Håkansson, Tjellström and Rosenhall, 1985; Sakai, Karino and Kaga, 2006; Bergin *et al.*, 2015; Chhan, McKinnon and Rosowski, 2017). The lack

of masking of the head vibration-induced potentials by intense noise (Fig. 4), suggest that these responses may be vestibular compound action potentials (Jones and Jones, 1996; Jones, Jones and Shukla, 1997; Brown, Pastras and Curthoys, 2017). This hypothesis is further supported by the notion that vestibular responses can be recorded prior to responses of the auditory system (Freeman *et al.*, 1999). Even if sound-induced vibrations of the egg would translate without any loss into vibrations of the zebra finch embryo, the level of the generated vibrations is orders of magnitudes too small to evoke responses of the vestibular system or to be detected by typical vertebrate vibrotactile senses (Fig. 5). Taken together, i) the lack of cochlear responses to bone conduction stimulation throughout postnatal development and at adulthood and ii) the absence of sufficiently strong vibrotactile stimuli to the egg generated by airborne sounds make it very unlikely that developing zebra finch embryos can perceive heat calls via detection of sound-induced vibrations.

The role of heat calls as a communication signal has been debated (McDiarmid, Naguib and Griffith, 2018; Mariette and Buchanan, 2019). They have instead been proposed to be a side product of thermally induced panting to increase ventilation (McDiarmid, Naguib and Griffith, 2018; Mariette and Buchanan, 2019; Pessato *et al.*, 2020). While heat calls have been recorded in several studies, the source level of the heat calls is currently unknown. It is therefore hard to evaluate the biological relevance of the used experimental playbacks because heat calls may be much softer *in vivo*. How the heat call playbacks have affected the embryos in the earlier studies remains to be further tested and reproduced.

While the sensitivity of the zebra finch auditory system to click stimuli is similar at 10 DPH and at adulthood, other aspects of the ABR continue to change. For example, the response latency of the auditory nerve (wave I) shortens past 20 DPH. Furthermore, the responses generated by the more upstream neural populations of the auditory system (wave II and above) appear to go through major changes after 10 DPH (Fig. 1c). Based on our findings, we believe that the peripheral structures and sound encoding circuits of the zebra finch auditory system continue to develop for several weeks after hatching. Similar observations have been made on the well-studied precocial chicken. While chicken auditory nerve sensitivity at hatching is close to what is observed at adulthood, the temporal coding ability of the auditory nuclei - the ability to respond to high stimulus rates - increases post-hatch (Saunders, Coles and Gates, 1973;

Saunders, Gates and Coles, 1974; Tucci and Rubel, 1990). Furthermore, the response characteristics of the auditory nerve fibers and the latencies of the ABR show postnatal changes (Katayama, 1985; Manley *et al.*, 1991; Jones, Jones and Paggett, 2006). The periphery of the chicken auditory system also continues to structurally mature for several weeks after hatching (Ryals, Creech and Rubel, 1984; Cotanche and Corwin, 1991; Cohen, Hernandez and Saunders, 1992; Jones, Jones and Paggett, 2006). Surprisingly, we know little about these changes in zebra finches. Experimental work indicates that zebra finches learn song only after 25 DPH, the onset of sensory learning (Arnold, 1975; Roper and Zann, 2006; Braaten, 2010; Gobes, Jennings and Maeda, 2019). The striking co-occurrence of this onset with the maturation of the auditory nerve responses (Fig. 1) suggests that the onset of song learning in zebra finches may be gated by the developmental maturation of the auditory system. It would be interesting to investigate this possibility in other songbird species where auditory system development has also been studied, and sensory and sensorimotor phases are more separated, such as e.g. budgerigars, and the characterization of motor learning is on the way (Zhao *et al.*, 2023).

Interestingly, all currently known vocal learners (songbirds, parrots, hummingbirds, bats, seals, humans, whales) seem to be altricial species. This raises the question if altriciality and particularly parental care could have been the key that unlocked the ability for vocal learning from ancestral precocial birds.

METHODS

Animals

Local, captivity-bred zebra finches (*Taeniopygia guttata*, order *Passeriformes*) were used in this study. Zebra finches were kept and bred indoors in temperature- and moisture-controlled group aviaries (100-to-200 individuals) or pairwise in breeding cages supplemented with nesting boxes. Lights in the aviaries were on a 12/12 hour light/dark photoperiod. Zebra finches were given water, food, cuttlefish bones, and nesting material *ad libitum*. Nesting boxes were monitored daily for accurate aging of the hatched chicks. The day when a given zebra finch chick was observed to have hatched in the morning was defined to be post-hatch day 0 (0 DPH). Hatched chicks were recognized from each other by making individualized, non-invasive down feather cuts until the usage of leg bands was feasible at DPH 10. Animal experiments were approved by the Danish National Animal Experimentation Board (Dyreforsøgstilsynet) under license number 2019-15-0201-00284.

Anesthesia

Preparation: 30-to-60 minutes prior to recording, 20 DPH-to-adult zebra finches were removed from their breeding cage or aviary, brought to the test room in a transport cage, and were fasted to minimize the reflux of fluid/food during anesthesia. Younger zebra finches were moved to the test room in a warmed transport box and were not fasted. Zebra finches were weighed prior to the induction of anesthesia.

Dosing: Anesthesia was induced with an intrapectoral injection of ketamine (Ketaminol Vet., 100 mg/ml, MSD Animal Health, NJ, USA) in combination with xylazine (Rompun vet., 20 mg/ml, Bayer Animal Health GmbH, Leverkusen, Germany). 20 DPH-to-adult zebra finches were administered 50 µg of ketamine and 10 µg of xylazine per gram of body weight. 2-to-10 zebra finches were given 50 µg of ketamine and 5 µg of xylazine per gram of body weight. After one hour, a supplementary dose (75% of the induction dose) was administered. In some zebra finches, a clear liquid - possibly due to fluid reflux or due to increased production of oral

secretions - was noticed in their mouths within 10-to-15 minutes from anesthesia induction. To avoid airways from being obstructed, a small piece of paper tissue was inserted to the side of the mouth to drain the liquid.

Air conduction -induced auditory brainstem responses (AC-ABRs)

Recording room: All recordings were performed by placing the zebra finch inside an audiometric room (T-room, C-A TEGNÉR AB, Bromma, Sweden) equipped with a self-built vibration isolation table with a padded surface. The walls of the room were padded with sound absorbing wedges (Classic Wedge 30, EQ Acoustics, UK).

Maintenance of body temperature: The body temperature of the animals was maintained by placing a heating pad warmed by circulating water (TP702, Gaymar Instruments Inc., Orchard Park, NY, USA) underneath them and placing a small cloth on top of their body. Body temperature monitoring was performed with a J-type temperature probe connected to a thermocouple measurement device (NI USB-TC01, National Instruments). The tip of the probe was coated with Sylgard (184 Silicone Elastomer Kit, Dow Europe GMBH, Wiesbaden, Germany), covered with vaseline, and was placed withing the cloaca. With 2-to-10 DPH zebra finches where the cloaca was too small for probe insertion the probe was placed under the wing.

Head placement: The head of the zebra finch was slightly elevated by placing it on top of wax mound (Surgident Periphery Wax, Kulzer, IN, US). The ends of a wedge-shaped notch on the wax were slightly pressed together so that the wax held the lower mandible of the zebra finch in place. The auricular feathers covering the left external auditory meatus in mature zebra finches were cut with scissors to allow unobstructed stimulation of the auditory system. Prior to electrode placement, the appropriate depth of anaesthesia was confirmed by the lack of responsiveness to a toe-pinch.

Electrodes: Three subdermal needle electrodes were placed subcutaneously on the head of the animal in the following configuration (Fig. 1A): (1) Active electrode: Dorsal to the left auditory meatus, pointing towards to the top of the head. (2) Inverting electrode: On top of the head along the midline, pointing towards the bill; (3) Ground electrode: Dorsal to the right auditory meatus, pointing towards to the top of the head. Of note, to compare these recordings to studies where the

positions of the active and inverting electrodes are the opposite one must invert the recorded signal. The impedances of the electrodes were measured (RA4LI, Tucker-Davis Technologies, FL, USA) to be below 1 kOhm in 2-to-10 DPH zebra finches and below 3 kOhm in 20 DPH-to-adult zebra finches.

Recording devices: Recorded evoked potentials were passed from the electrodes to a headstage (RA4LI) in series with a preamplifier (RA4PA) and with a digital signal processor (RM2, all from Tucker-Davis Technologies, FL, USA). RM2 was controlled with custom-written software QuickABR (Brandt, Brande-Lavridsen and Christensen-Dalsgaard, 2018). Recorded responses were digitized with a sampling rate of 25 kHz (16 bits).

Sound stimulation: Sound stimulation was generated in the RM2 controlled by QuickABR, amplified (Cambridge Audio, Azur 740A Integrated Amplifier, London, UK), and emitted from a loudspeaker (Wharfedale Diamond 220, Wharfedale Ltd., Huntingdon, UK). The loudspeaker was string-suspended 25 cm away from the animal, directly facing the left auditory meatus. Sound reflections were minimized by placing the loudspeaker at an ~45-degree angle relative to the closest wall of the recording room. To calibrate the loudspeaker, a ½" microphone (G.R.A.S. microphone type 26AK, G.R.A.S., Holte, Denmark) was suspended at the position of the head of the animal and connected to a microphone amplifier (G.R.A.S. Power Module Type 12AA, Gain 0, Linear filtering, Direct mode select = Ampl.). The microphone itself was calibrated using an acoustical calibrator (type 4321, Output: 1 kHz at 94 dB re. 20 µPa, Brüel & Kjær). Calibration of the loudspeaker was controlled by QuickABR.

Stimuli: AC-ABRs were elicited with broadband clicks (20 µs) and 25 ms-long tonebursts at 0.25 kHz, 0.5 kHz, 1 kHz, 2 kHz, 3 kHz, 4 kHz, 6 kHz, and 8 kHz. To minimize "spectral splatter" caused by abrupt on/offsets of stimuli, the rise/fall time (5 ms, $\cos^2$-gated) of the tonebursts was selected to accommodate at least one full cycle of the stimulus with the lowest frequency. To further increase the frequency specificity of the stimulus, the plateau time (15 ms) was selected to allow at least 3 full cycles of the stimulus with the lowest frequency. Additionally, 5 ms-long tonebursts (1 ms rise/fall time, $\cos^2$-gated) were collected from adult zebra finches. Stimuli were sampled at 25 kHz and repeated at a rate of 25 Hz to collect average responses consisting of 400 individual responses. Every second presented individual stimulus was inverted to reduce microphonic effects. The maximal sound pressure level (SPL) used in this study was selected to

be 95 dB SPL as it can be viewed as being physiologically relevant. Click amplitudes are stated as the root mean square (RMS) dB value of a sinusoid with the same peak amplitude as the click (peak equivalent dB).

Recording paradigm: The stability of the recorded responses was monitored throughout the recording session by recording a 95 dB SPL click-evoked AC-ABR periodically between each stimulus type. A specific stimulus recording was accepted into further analysis if the amplitude of the click-evoked AC-ABR wave I had changed less than 25% from the most temporally stable amplitude of that recording session. To minimize test order bias, the recording order of stimuli with varying frequencies was randomized. Furthermore, BC-ABRs were collected to prior to AC-ABRs in some animals.

Head vibration-induced potentials and bone conduction-induced ABRs (BC-ABRs)

Setup and calibration: A vibrometer was attached to a piston (4501 A 30424, Brüel & Kjær, Copenhagen) and connected to a preamplifier (1704-A-002, Gain 100x, Lin filter, Brüel & Kjær, Copenhagen). The piston was then attached to an electrodynamic vibration exciter (Mini Shaker Type 4810, Brüel & Kjær, Copenhagen) surrounded by a grounded wire mesh. The system was calibrated by using a vibration calibrator (TYPE 4294 Portable Vibration Calibrator, 10 m s^{-2} standard acceleration level at 159.15 Hz, Brüel & Kjær, Copenhagen). Calibration was controlled by QuickABR. Maximal tested acceleration level was selected to be 10 m s^{-2} (100 dB re 0.1 mm s^{-2}) to ensure animal safety during recordings and as the value is close to the physiologically relevant gravitational acceleration (1 g-force).

Procedure: The vibrational exciter was lowered on a holder so that the attached piston made physical contact with the proximal end of the upper mandible of the anesthetised zebra finch. The piston was perpendicular to the culmen of the upper mandible. A small drop of tissue adhesive (VetBond, 3M) was placed between the piston and the mandible to secure their physical contact. Head vibration-induced potentials were elicited with the vibration exciter with same stimulus types and recorded with the same electrodes/devices as AC-ABRs (see above).

Control experiments: After a recording session, used vibrational stimuli were re-recorded in the absence of probe-to-head contact by slightly lifting the probe off from the bill. To test whether

an intense AC-stimulus could mask the head vibration-induced potentials, white noise was played at 95 dB SPL simultaneously while recording the vibrational responses. A subset of animals (N = 3) was used to determine whether the recorded responses are dependent on the animal being alive. At the end of the specific recording session the animal was killed with a lethal overdose of ketamine-xylazine without removing it from the recording setup. *Ex vivo* responses were recorded after the animal visibly had stopped breathing. The SPL of the airborne sounds generated by the vibration exciter were recorded with the same devices as the AC-ABR stimuli were calibrated with. The used microphone was placed next to the vibration exciter at the location where the ear of the tested zebra finch would reside.

Egg vibration measurements with laser doppler vibrometry

Zebra finch eggs were removed from their nest and placed in an upright position on a dental wax covered platform at the centre of an anechoic room. The anechoic room was custom-made with its walls and floor covered with 30 cm rockwool wedges. The sample platform was mounted on a heavy tripod which also held a laser Doppler vibrometer (OFV-5000 with OFV-505 sensor, Polytec, Waldbronn, Garmany) attached on a manipulator. The laser was pointed at the center of the side of the egg. Strong reflections were directly obtained from the egg surface so adding reflectors was not necessary. Sound-induced vibrations of the egg were induced with a speaker (JBL IG, Northridge, CA, USA) placed next to the laser vibrometer. A microphone (½ inch G.R.A.S., Brüel & Kjær) was placed few millimeters above the egg and was used for speaker calibration. The egg was stimulated with sound frequency sweeps (0.25-to-10 kHz) at 84 dB SPL. The sweeps were generated with Tucker-Davis system 2 hardware (Tucker-Davis Technologies, TDT, Alachua, FL, USA) controlled by custom-written software DragonQuest (Christensen-Dalsgaard and Manley, 2008). The signal was deconvoluted by dividing the spectrum of the sweep with the measured transfer function of the speaker. The analog laser signal of the laser vibrometer was digitized using an A–D converter (AD2, TDT) and averaged over 100 presentations. During recordings, the laser signal was monitored using an oscilloscope and a vibrometer controller (Polytec OFV-5000). Stimulation and recording were controlled by DragonQuest.

Data analysis and visualization

Data was visualized and analysed with custom scripts in MATLAB R2022B (Mathworks). Figures were edited with Illustrator CS6 (Adobe).

Threshold determination: For AC-ABR and head vibration-induced potential threshold detection, the stimulus level was lowered from the maximal value in 5 dB steps. For the reported thresholds to reflect the average sensitivity of zebra finches capable of detecting the given stimulus, only zebra finches that displayed a detectable (both visually and above the signal-to-noise ratio of 2) AC-ABR or a head vibration-induced potential at the maximal used stimulation level are included in the analysis. The response threshold was designated to be 2.5 dB below the last observable response-generating stimulus level (*arrow* in Fig. 1f).

Signal-to-noise ratio calculation: The recording prior to stimulus onset was used to calculate the noise RMS. Signal-to-noise ratio (S/N) was determined by dividing the RMS value of the signal (*blue window*) by the RMS of the recording prior to stimulus onset (*red window*, Figs. 1c., 2a, 2b, 4a, 4b, and Supplementary Fig. 2a). The analysis window to determine the signal RMS for head vibration-induced potentials was positioned so that the AC-ABR responses evoked by the sounds generated by the vibration exciter would not contaminate the results (Fig. 4a).

Response latency and amplitude calculation: Response latency was calculated as the time difference between the first negative deflection of the AC-ABR (designated as wave I) and the arrival time of the sound at the ear (Fig. 1c). The amplitude of the wave I (reflecting the activity of the auditory nerve) was calculated as the potential difference between the first negative peak and the following positive trough that together formed the most prominent component of the response (Fig. 1c). Of note, a second negative peak/deflection between the first peak and the trough existed. This peak appeared to merge with the first negative peak at soft SPLs as reported for AC-ABRs of bald eagles and red-tailed hawks (McGee *et al.*, 2019).

Statistics

To test for main effects, we used linear mixed effects models. We sequentially compared models by adding hypothesised effects as fixed effects one-by-one, starting from a null model and ending with full interaction models. For all age comparisons, age was treated as an ordinal rather

than a continuous variable to account for discrete timepoints of measurements and to allow for post-hoc pairwise comparisons between ages. All models had the individual animals as random effect. We used chi-square tests, Akaike Information Criterion (AIC), and Bayes Information Criterion (BIC) as criteria for model comparison. For models with significant main effects, we then used estimated marginal means for post-hoc comparisons. We used Bonferroni p-value correction to account for multiple comparisons. In all figures, we denote significance levels as follows: *, p(Bonf.) = 0.05; **, p(Bonf.) = 0.01; ***, p(Bonf.) = 0.001. All statistical testing was carried out in R 4.2.1 (R Core Team, 2024) We used the lme4 package (Bates *et al.*, 2015) for mixed effects modelling and emmeans package (Lenth, 2024) for post-hoc comparisons.

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END NOTES

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Author contributions statement

T.A., J.C.D., and C.P.H.E. conceptualized experiments; T.A. collected and analyzed the data for figures 1-to-4. T.A. and J.C.D. collected and analyzed the data for figure 5. T.A. and C.P.H.E. wrote the draft of the manuscript; T.A., J.C.D., and C.P.H.E. wrote the manuscript. T.A. and C.P.H.E. acquired funding.

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Competing interests

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