Progressive postnatal hearing development limits early parent-offspring vocal communication in the zebra finch

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Abstract

Acoustic communication relies critically on the receiver's ability to hear. In precocial bird species hearing can already be functional during embryonic stages in the egg, while in altricial bird species hearing typically starts after hatching. Recent research suggests that zebra finch embryos, despite being altricial, have functional hearing already in the egg to engage in parent-embryo acoustic communication and in anthropogenic noise detection. However, their auditory sensitivity during early development remains unknown. Here, we measure auditory brainstem responses over early postnatal development and show that zebra finch hatchlings are deaf even to loud, broadband sounds with hearing responses emerging between 4-8 days after hatching. Auditory sensitivity develops progressively and reaches adult levels by day 20, contradicting the notion of early parent-to-embryo communication in zebra finches. Additionally, egg vibrations induced by sound remain far below detection thresholds of vibrotactile senses. The striking timing coincidence between maturation of the peripheral auditory system and the onset of song learning suggests that hearing functionality may gate the onset of vocal learning in zebra finches.

Introduction

The ability to detect sound is critical for vocal communication, species recognition, predator detection, and survival across the vertebrates (***Nordeen and Nordeen, 1992***; ***Heaton et al., 1999***; ***Zevin et al., 2004***; ***Ladich and Winkler, 2017***). In birds, precocial species that are able to move around after hatching show some hearing function already inside the egg (***Gottlieb, 1965***; ***Saunders et al., 1973***; ***Konishi, 1973***). This enables embryo-to-embryo (***Vince, 1966***; ***Woolf et al., 1976***; ***Schwagmeyer et al., 1991***; ***Noguera and Velando, 2019***) and parent-to-embryo communication that is critical to imprinting on species-specific sounds (***Gottlieb, 1965***; ***Grier et al., 1967***; ***Hess, 1972***). In contrast, hearing function in altricial birds is thought to mature predominantly after hatching with little to no functional hearing during embryonic stages (***Woolley, 2017***).

Remarkably, recent research directly challenges this concept and suggests that altricial songbirds can detect soft sounds while still in the egg (from here onwards referred to as embryos). Zebra finch embryos supposedly are epigenetically guided to adapt to high temperatures by their parents high frequency "heat calls" (***Mariette and Buchanan, 2016***; ***Mariette et al., 2018***; ***Katsis et al., 2018***; ***Mariette and Buchanan, 2019***; ***Pessato et al., 2020***; ***Udino et al., 2021***; ***Katsis et al., 2021***; ***Udino and Mariette, 2022***; ***Katsis et al., 2023***) (from here onwards referred to as heat whistles)

and can detect anthropogenic noise as prehatching noise exposures reduce embryonic survival and reproductive success in adulthood (***Meillère et al., 2024***). Furthermore, fairy wren embryos supposedly learn elements of the female incubation call already at 10-12 days after fertilization (***Colombelli-Négrel et al., 2012***, ***2014***, ***2016***; ***Kleindorfer et al., 2024***). The above interpretations critically depend on the ability of these embryos to detect soft level sound in the egg and after hatching. However, it is currently unknown whether these species can detect sound this early in their development.

The zebra finch is a major animal model system for studying the neural circuitry underlying hearing-guided behaviours including vocal imitation learning. However, we know surprisingly little about development and function of the auditory organ in this species. In singing males, the onset of sensory and sensorimotor learning starts around 25 days-post-hatch (DPH) and continues to adulthood at ~90 DPH (***Immelmann, 1967***; ***Böhner, 1990***; ***Roper and Zann, 2006***; ***Braaten, 2010***; ***Gobes et al., 2019***). Aligned with the onset of this behaviour, the overall hearing sensitivity is comparable to adult levels at 20 DPH with a frequency range from 0.25 to 8 kHz and highest sensitivity around 3 kHz (***Amin et al., 2007***; ***Okanoya and Dooling, 1987***; ***Yeh et al., 2023***). However at 10 DPH – the earliest age measured – hearing sensitivity is reduced by 25 dB compared to 20 DPH and is thus ~20-fold lower (***Amin et al., 2007***). Because hearing sensitivity in zebra finches increases progressively in late postnatal development - as in other bird species (***Rebillard and Rubel, 1981***; ***Katayama, 1985***; ***Jones et al., 2006***; ***Brittan-Powell and Dooling, 2004***; ***Köppl and Nickel, 2007***; ***Kraemer et al., 2017***) - their ability to detect sounds as embryos and as early hatchlings may be severely compromised. Furthermore, recent work shows that heat whistles are extremely soft sounds *in vivo* (~34 dB re. 20 µPa at 10 cm) (***Anttonen et al., 2025***). Therefore, zebra finch embryos would require highly sensitive hearing function to detect such soft sounds. However, we lack conclusive evidence on the hearing capabilities of juvenile zebra finches.

Here we test the hypothesis that zebra fiches can hear during early postnatal development by measuring auditory brainstem responses (ABRs) that allow hearing threshold estimation when behavioural training is not possible (***He et al., 2008***). Furthermore, we test whether loud sound fields can induce egg vibrations of sufficient magnitude for vibrotactile detection. We show that the earliest ABRs evoked by intense, broadband sounds could not be detected on 2 DPH, but earliest at 4-8 DPH. Responses to frequency specific sounds develop later in a progressive, low-to-high frequency manner. ABR sensitivity to clicks matures to adult-like levels at 10 DPH but the response amplitude continues to increase beyond 20 DPH. Airborne sounds induce low amplitude vibrations in eggs that are far below typical avian and mammalian vibrotactile detection thresholds. Together, our findings show that zebra finch embryos and early hatchlings are functionally deaf and provide evidence against the hypothesis that they can detect sound stimuli via hearing or via vibrotactile senses. Our data furthermore show that auditory nerve responses continue to mature past 20 DPH, suggesting that the maturation of hearing limits the onset of vocal learning in zebra finches.

Results

Responses to loud clicks appear during the first postnatal week

To assess the development of zebra finch hearing sensitivity in early postnatal development we quantified the responses of auditory pathway neurons to sound stimuli by recording ABRs (**Fig. 1A**). 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 ABRs, thus providing maximal sensitivity for threshold detection.

Clicks induced robust neural potentials in adult zebra finches (**Fig. 1C-E**). These potentials were not recording artefacts as they occurred only when the animal was alive (N = 3, **Fig. 1C**). None of the tested animals displayed click-evoked 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. 1D,E**). However, click-evoked ABRs

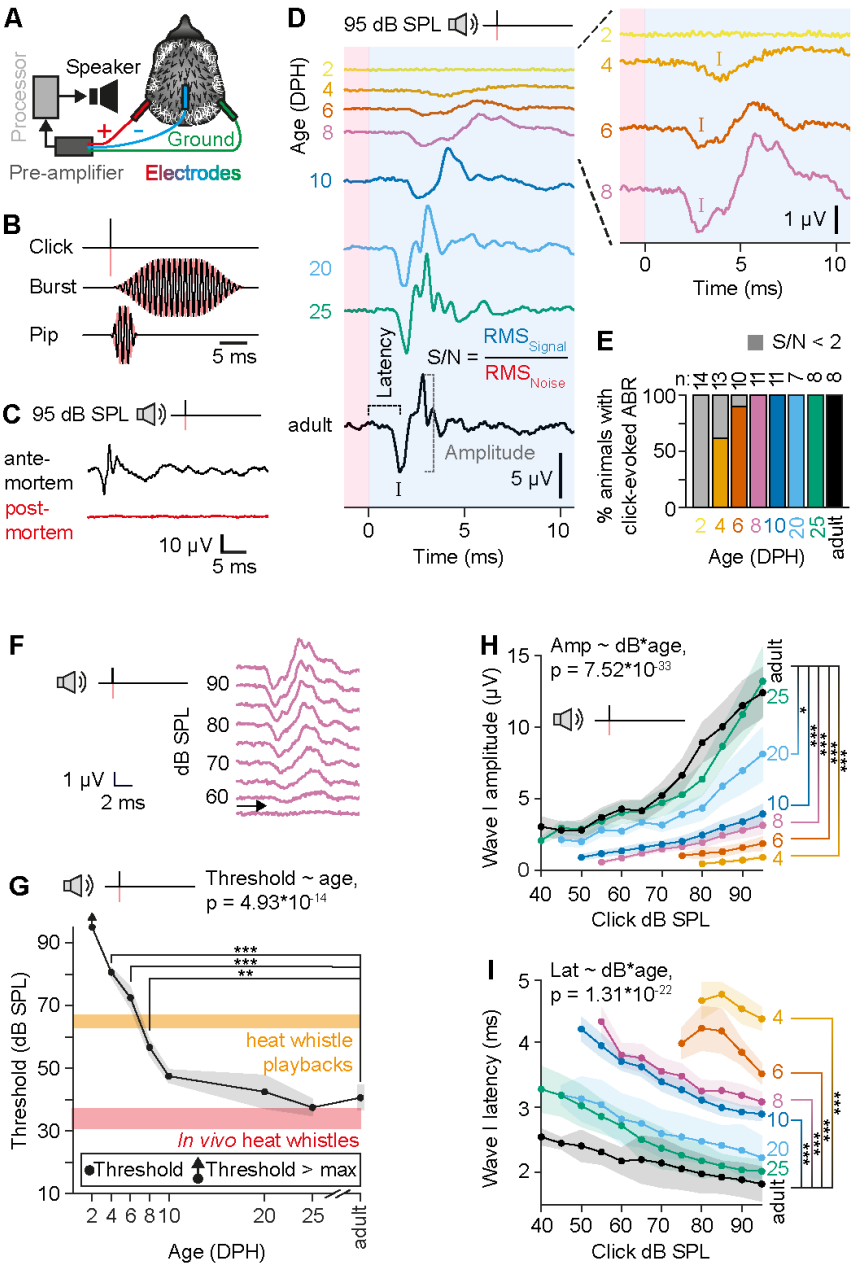

Figure 1. Click-evoked ABRs appear and gradually mature after hatching in zebra finches. (**A**) ABR recording setup. (**B**) Used sound stimuli. *Red*, phase inverted signal. (**C**) Representative, 95 dB SPL click-evoked ABR *ante-* (*black*) and *post-mortem* (*red*). (**D**) Representative ABRs to 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*. (**E**) Percentage of animals that exhibited a detectable (S/N > 2) click-evoked ABR. (**F**) Detection thresholds were measured with a set of clicks with decreasing 5 dB SPL steps. *Arrow* indicates the determined threshold for this 8 DPH example. (**G**) Average click thresholds at different DPH ages. *Orange box* indicates heat whistle playback levels used in previous studies (***Katsis et al., 2018***). *Red box* indicates *in vivo* heat whistle levels at 10 cm distance (***Anttonen et al., 2025***). (**H**) Click-evoked ABR wave I amplitude and (**I**) latency over SPL. Age groups are *color-coded* in D-I. *Shaded areas* in H-I = s.e.m.

became observable in some animals during the first postnatal week (S/N > 2; 4 DPH, n = 5/13; 6 DPH, n = 9/10; **Fig. 1D,E**). Subsequently, all tested animals consistently demonstrated click-evoked ABRs from the second postnatal week onwards (S/N > 2; 8 DPH, n = 11; 10 DPH, n = 11; 20 DPH, n = 7; 25 DPH, n = 8; Adult, n = 8; **Fig. 1D,E**). Thus, sounds at loud, yet physiologically relevant SPLs do not evoke ABRs in the first days after hatching and in the majority only after one week.

Click-evoked ABR thresholds are adult-like at 10 DPH

To estimate hearing sensitivity thresholds over postnatal age, we recorded series of click-evoked ABRs at 5 dB downward steps from 95 dB SPL (**Fig. 1F**). Hearing thresholds are strongly affected by the age of the animal ($F(6) = 25.22$, $p = 4.93*10^{-14}$; **Fig. 1G**, **Table S1**). Click-evoked 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, $t = 5.92$, $p = 1.39*10^{-6}$; 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. 1G**, **Table S1**). Click thresholds are as high as 80.6 ± 1.6 dB SPL on 4 DPH. Thus, zebra finch hearing sensitivity increases rapidly but gradually during the first two postnatal weeks.

ABR wave I gradually reaches maturity 25 days after hatching

The click-evoked ABR consists of multiple wave peaks and troughs that reflect the subsequent activations of various neural populations of the auditory pathway. The first wave (wave I in **Fig. 1D**) is considered to correspond 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 the amplitude and the latency of wave I over postnatal development.

In adults, the amplitude of wave I increases significantly with click SPL (**Fig. 1H**). Age 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. 1H**, **Table S2**). The steepness of the amplitude-SPL slope gradually increases with age and 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. 1H**, **Table S2**).

The latency of wave I decreases with increasing click SPL (**Fig. 1I**). Furthermore, age 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. 1I**, **Table S3**). 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, $t = 2.24$, $p = 0.18$; 25 DPH vs. adult, $t = 1.67$, $p = 0.61$; p-values are Bonferroni-corrected; **Fig. 1I**, **Table S3**).

Taken together, the synchronized output of the auditory nerve increases in sensitivity and reduces in latency postnatally progressively from 4 DPH until reaching maturity at 20-25 DPH. Such gradual increase is consistent with postnatal hearing development in budgerigars, mice, and humans (***Brittan-Powell and Dooling, 2004***; ***Hecox and Galambos, 1974***; ***Henry and Haythorn, 1978***).

Hearing sensitivity develops progressively from low to high frequencies

When in a hot environment, zebra finch parents emit heat whistles that have been proposed to epigenetically guide their unhatched offspring to adapt to high temperatures (***Mariette and Buchanan,***

2016; ***Mariette et al., 2018***; ***Katsis et al., 2018***; ***Mariette and Buchanan, 2019***; ***Pessato et al., 2020***; ***Udino et al., 2021***; ***Katsis et al., 2021***; ***Udino and Mariette, 2022***; ***Katsis et al., 2023***). These whistles have been reported to have high fundamental frequencies (7-10 kHz) when compared to zebra finch vocalisations (***Mariette and Buchanan, 2016***). As hearing sensitivity typically develops in a low-to-high frequency order (***Brittan-Powell and Dooling, 2004***; ***Kraemer et al., 2017***; ***Ehret, 1976***; ***Ehret and Romand, 1981***), such high frequency sounds may be challenging to be detected by the immature auditory system.

To study the postnatal development of frequency-specific hearing sensitivity we tested whether loud (95 dB SPL) tone bursts centred at frequencies between 0.25-to-8 kHz elicit detectable ABRs. Tone burst-evoked ABRs are first detectable on 8 DPH at 1 kHz (**Fig. 2A** and **Fig. S1A**). As observed with click stimuli, the amplitude of tone burst-induced ABRs increases with age (**Fig. 2A**, **Fig. S1B, Table S2**; Age as a predictor of amplitude compared to null model: $\chi^2(3) = 6.11$, $p = 0.11$). The latency of the response decreases significantly with age (**Fig. 2a**, **Fig. S1c, Table S2**; Age as predictor of latency compared to null model: $\chi^2(3) = 33.72$, $p = 2.27*10^{-7}$). Latencies are significantly longer at 8-to-10 DPH than at adulthood (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 (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; **Fig. S1C** and **Table S2**). During the following weeks of postnatal development, tone burst-induced ABRs became detectable at 0.5-to-4 kHz (**Fig. S1A**). No responses were detected at higher frequencies (**Fig. 2B,C**; **Fig. S1A**). Taken together, the maturation of hearing sensitivity in zebra finches progresses in a low-to-high frequency order as is also observed for example in mice (***Ehret, 1976***), cats (***Ehret and Romand, 1981***), and in other birds (***Brittan-Powell and Dooling, 2004***; ***Köppl and Nickel, 2007***; ***Kraemer et al., 2017***).

In vivo heat whistles are below adult hearing threshold levels

Most birds have strongly decreasing hearing sensitivity above 5 kHz (***Okanoya and Dooling, 1987***; ***Yeh et al., 2023***; ***Rebillard and Rubel, 1981***; ***Brittan-Powell and Dooling, 2004***; ***McGee et al., 2019***). Given the 7-10 kHz frequency range of heat whistles, it is unknown if even adult zebra finches can readily detect them.

To test this, we analysed adult hearing sensitivity for higher frequency sound stimuli in more detail. Importantly, tone bursts evoke ABRs only in about half of the adults (**Fig. S1A**) and the detection thresholds of > 60 dB SPL are high. This indicates that while being more frequency specific, tone bursts failed to recruit auditory neurons to fire concurrently enough to generate detectable ABRs. Therefore, we next used shorter 5 ms-long tone pips (**Fig. 1B**) that have a broader spectral distribution of acoustic energy compared to tone bursts and produce a more focused temporal response (***Lauridsen et al., 2021***). Indeed, the SPL matched tone pips produced much more prominent ABRs (**Fig. 2C** and **Fig. S2A**) that were readily detected in all tested adults around their most sensitive hearing range of 1-to-4 kHz (***Amin et al., 2007***; ***Okanoya and Dooling, 1987***; ***Yeh et al., 2023***) (**Fig. S2B**). Detection thresholds for tone pips are significantly more sensitive than those of tone bursts with ~20 dB SPL (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: $\chi^2(1) = 10.27$, $p = 0.001$, **Table S2**). Importantly, the tone pip-based audiogram has similar shape and is shifted ~10-to-20 dB SPL upwards compared to behavioural thresholds (***Okanoya and Dooling, 1987***) (**Fig. 2C**), which is consistent with previous studies (***Stapells, 2000***).

Based on these audiograms, we can conclude that even for adult zebra finches, heat whistles at playback levels of 62-to-67 dB SPL at the ear (***Katsis et al., 2018***) and at *in vivo* levels of 33.9±3.3 dB SPL at 10 cm (***Anttonen et al., 2025***) are just on or below their detection limit. More importantly, as hearing sensitivity is much lower in less developed hatchlings (**Fig. 1 and 2**), these data further suggest that early hatchlings cannot hear high-frequency heat whistles.

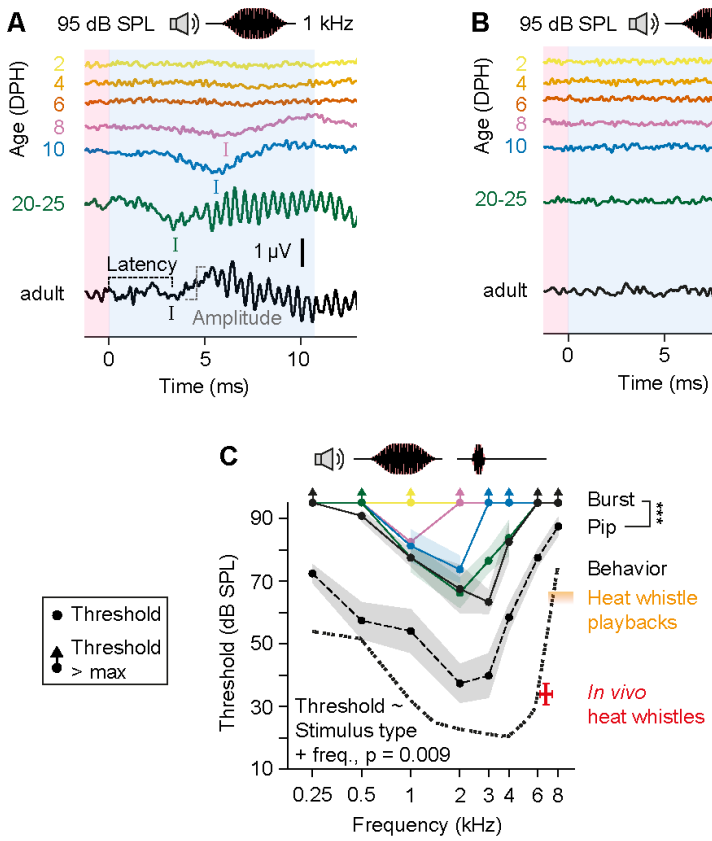

Figure 2. Sound frequency sensitivity matures over postnatal development. (**A**) Representative ABRs to 1 kHz and (**B**) 8 kHz tone burst stimuli over postnatal development. (**C**) Zebra finch ABR thresholds obtained with tone bursts (*solid lines*) and with tone pips (*dashed line*). *Dotted line* displays a behavioural threshold reported by Okanoya and Dooling (1987). *Shaded areas* = s.e.m. The level of heat whistle playbacks used in previous studies (***Katsis et al., 2018***) (*orange box*) and the level of *in vivo* heat whistles at 10 cm distance (***Anttonen et al., 2025***) (*red lines*) are just on or below the adult hearing threshold.

Sound-induced egg vibrations are below detectable levels

Because our data strongly suggests that embryonic zebra finches cannot detect soft, high frequency heat whistles as airborne sounds via their auditory system, we next tested if airborne sounds can induce zebra finch eggs to move at sufficient velocities to be detected as vibrations (***Håkansson et al., 1985***; ***Sakai et al., 2006***; ***Bergin et al., 2015***; ***Chhan et al., 2017***; ***Mountcastle et al., 1972***; ***Hörster, 1990***). We played sound frequency sweeps to eggs while simultaneously recording the sound-induced vibrations of the egg with a laser vibrometer (**Fig. 3A**). The vibration velocity transfer function shows that eggs vibrate at velocities around -85 to -55 dB re 1 mm s^{-1} when exposed to a loud 94 dB SPL frequency sweep between 0.25-to-10 kHz (**Fig. 3B**). Within the frequency ranges associated with the most sensitive hearing of zebra finches (1-4 kHz, *green*) and with heat whistles (7-10 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 (**Fig. 3B,C**). We calculated the corresponding vibration velocity levels induced by SPLs used during experimental heat whistle playbacks to zebra finch eggs (62-to-67 dB SPL) (17) to be between -99.4 and -96.2 dB re 1 mm s^{-1}, respectively (**Fig. 3D**). The obtained vibration velocities are approximately 10-to-40 dB lower than typical vertebrate bone conduction thresholds (***Håkansson et al., 1985***; ***Sakai et al., 2006***; ***Bergin et al., 2015***; ***Chhan et al., 2017***) and 90 dB lower than what human (***Mountcastle et al., 1972***) and bird (***Hörster, 1990***) vibrotactile senses typically can detect (**Fig. 3D**). Detection of *in vivo* heat whistles is even less likely as they are at least 27 dB softer compared to the playbacks levels (***Anttonen et al., 2025***). In con-

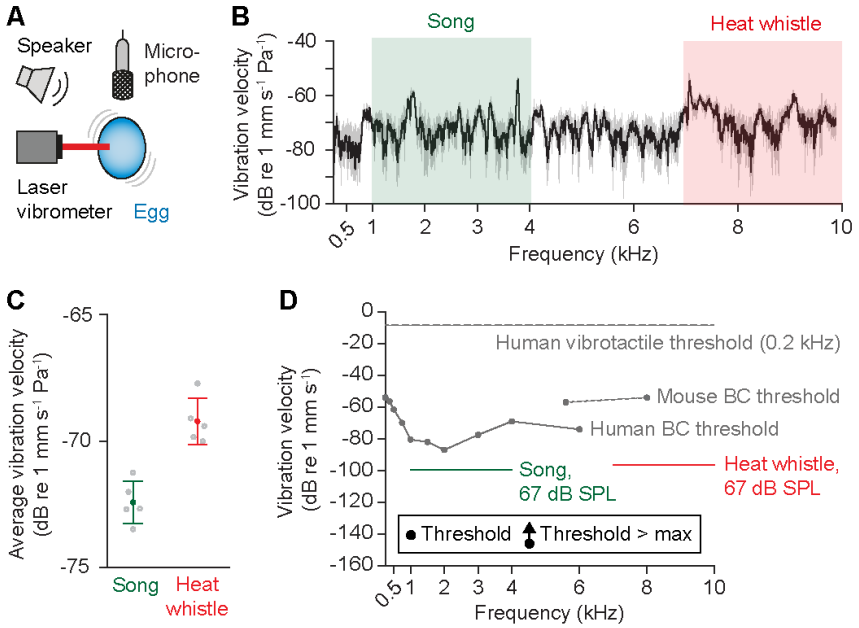

Figure 3. Intense sound stimulation induces zebra finch eggs to vibrate at very low velocities. A Setup used to measure sound induced vibrations of eggs. **B** Average (N=5) vibration velocity transfer function of a zebra finch egg during a 94 dB SPL sound sweep between 0.25-to-10 kHz. *Gray area* marks the standard deviation. *Green* and *red areas* display the frequency windows related to the best hearing sensitivity of zebra finches and to heat whistles, respectively. **C** The average egg vibration velocities induced by a 94 dB SPL sounds at frequencies associated with song (*green*) and with heat whistles (*red*). Error bars = s.d. **D** The average egg vibration velocities caused by 67 dB SPL sounds plotted together with thresholds for human (***Håkansson et al., 1985***) and mouse bone conduction hearing (***Chhan et al., 2017***) and for human vibrotactile threshold at 0.2 kHz (***Mountcastle et al., 1972***).

clusion, 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 known avian and mammalian vibrotactile senses.

Discussion

We show that zebra finch early hatchlings are functionally deaf, and that hearing functionality gradually increases over postnatal development. The synchronized output of the auditory nerve is first detectable at 4-8 DPH after which hearing sensitivity gradually increases until reaching adult levels at 20-25 DPH. This increase in hearing sensitivity over postnatal development from low-to-high frequencies is found in both altricial and precocial bird species (***Saunders et al., 1973***; ***Konishi, 1973***; ***Rebillard and Rubel, 1981***; ***Katayama, 1985***; ***Jones et al., 2006***; ***Brittan-Powell and Dooling, 2004***; ***Köppl and Nickel, 2007***; ***Kraemer et al., 2017***; ***Aleksandrov and Dmitrieva, 1992***; ***Lippe, 1994***) including zebra finches (***Amin et al., 2007***) - as well as in mammals (***Henry and Haythorn, 1978***; ***Ehret and Romand, 1981***,?). Therefore, the functional maturation of hearing in zebra finches follows a gradual, progressive pattern consistent with other birds and mammals.

The progressively increasing hearing sensitivity over postnatal development (**Fig. 1**) strongly suggests that zebra finch embryos have an even lower hearing sensitivity than early hatchlings. Even in precocial bird species that show auditory function already in the egg, auditory thresholds remain very high until a few days before hatching (***Saunders et al., 1973***; ***Rebillard and Rubel, 1981***; ***Lippe, 1994***; ***Saunders et al., 1974***). In precocial chickens, evoked responses to very loud sounds (>100 dB SPL) have been obtained around embryonic day 12 (chickens hatch at day 21) (***Saunders et al., 1973***), but ambient sound levels evoke responses around four days later (***Jones et al., 2006***). Importantly, these measurements involved experimentally opening the egg, clearing the auditory

meatus from fluids, and even drainage of fluids from the middle ear (***Rebillard and Rubel, 1981***) and thus may overestimate the natural hearing sensitivity. Although ABR provides easily accessible measurements of hearing sensitivity, the technique does not inform on the anatomical and neural processes underlying the gradual development of hearing sensitivity. In birds, these processes have been studied in most detail in chickens, where fluid unloading and maturation of middle ear structures causes significant improvements in hearing sensitivity during development (***Saunders et al., 1973***; ***Cohen et al., 1992***). The onset of evoked responses to very loud sounds furthermore coincides with synapse formation on hair cells of the inner ear (***Saunders et al., 1973***; ***Cohen and Fermin, 1978***; ***Caus Capdevila et al., 2021***). Furthermore, the response characteristics of the auditory nerve fibers and the latencies of the ABR show postnatal changes (***Katayama, 1985***; ***Jones et al., 2006***; ***Manley et al., 1991***). The periphery of the chicken auditory system also continues to anatomically mature for several weeks after hatching (***Cohen et al., 1992***; ***Ryals et al., 1984***; ***Cotanche and Corwin, 1991***). When these hallmark events occur in zebra finch development remains unknown. Further work is required to understand the physiological basis for postnatal development of hearing sensitivity in the zebra finch.

Our data provides evidence against the hypothesis that zebra finch embryos and early hatchlings can detect vibroacoustic stimuli via the auditory system or via vibrotactile senses. Our results therefore challenge previous interpretations that zebra finch parents use sound or vibration to communicate with their hatchlings and embryos (***Mariette and Buchanan, 2016***; ***Mariette et al., 2018***; ***Katsis et al., 2018***; ***Mariette and Buchanan, 2019***; ***Pessato et al., 2020***; ***Udino et al., 2021***; ***Katsis et al., 2021***; ***Udino and Mariette, 2022***; ***Katsis et al., 2023***). Particularly, the late maturation of high frequency hearing makes heat whistles (7-10 kHz) challenging to be detected by adults, let alone by developing zebra finches. The difficulty to detect high frequency signals is not unique to zebra finches; even precocial species that have hearing function prior to hatching have high response thresholds for sounds above 6 kHz as embryos (chicken: >120 dB SPL; mallard duck: >90 dB SPL) (***Saunders et al., 1973***; ***Katayama, 1985***). The established parent-embryo communication in 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***) which is consistent with limited high frequency hearing. An alternative explanation for the detection of heat calls by zebra finch embryos (***Mariette, 2024***) could be sound-induced vibrations of the egg or bone conduction hearing. However, even if sound-induced vibrations of the egg would translate without loss into vibrations of the embryo, our data show that the generated vibrations are orders of magnitudes too small to evoke responses from typical vertebrate auditory or vibratory senses (***Mountcastle et al., 1972***; ***Hörster, 1990***; ***Jones and Jones, 1996***; ***Freeman et al., 1999***). Taken together, our results provide evidence against the hypothesis that zebra finch embryos can hear sounds, and we consider it thus unlikely that adult zebra finches can communicate with their embryos via air-borne sound. How the heat whistle and anthropogenic noise playbacks have affected the embryos in the earlier studies (***Mariette and Buchanan, 2016***; ***Mariette et al., 2018***; ***Katsis et al., 2018***; ***Mariette and Buchanan, 2019***; ***Pessato et al., 2020***; ***Udino et al., 2021***; ***Katsis et al., 2021***; ***Udino and Mariette, 2022***; ***Katsis et al., 2023***; ***Meillère et al., 2024***) remains to be further tested. Importantly, the earlier work with heat whistle playbacks must be reproduced at physiologically relevant sound levels as they are ~27 dB too high when compared to *in vivo* heat whistles (***Anttonen et al., 2025***).

The postnatal maturation of hearing may also affect the onset and efficacy of vocal imitation learning. In zebra finches, sensory song learning starts on 25 DPH (***Immelmann, 1967***; ***Böhner, 1990***; ***Roper and Zann, 2006***; ***Braaten, 2010***; ***Gobes et al., 2019***). While the auditory sensitivity to click stimuli appears be mature at 10 DPH, the response amplitude of the auditory nerve (ABR wave I) increases past 20 DPH. Furthermore, ABR features that are generated by more upstream neural populations, e.g. wave II and later, appear to go through major changes after 10 DPH (**Fig. 1C**). These data suggest that both peripheral auditory nerve and downstream circuits of the zebra finch auditory system continue to develop for several weeks after hatching. In chickens, the temporal

coding ability of the auditory nuclei - the ability to respond to high stimulus rates – also increases post-hatch (***Saunders et al., 1973***, ***1974***; ***Manley et al., 1991***; ***Warchol and Dallos, 1990***). However, we know surprisingly little about these changes in zebra finches in early development (***Hong and Sanchez, 2018***) but they seem to continue well into the sensorimotor phase of song learning. The striking co-occurrence of the onset of the sensory phase of song learning with the maturation of the auditory nerve responses suggests that the onset of song learning in zebra finches may be gated by the developmental maturation of the auditory system. Furthermore, nascent, internal song templates acquired prior to the full maturation of hearing function may also require updating to match the changing target.

Methods and Materials

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 humidity-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 DPH 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.

Auditory brainstem responses (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 et al., 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 a 45° 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: 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 ABR periodically between each stimulus type. A specific stimulus recording was accepted into further analysis if the amplitude of the first click-evoked ABR wave 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. Three animals were killed with an overdose of ketamine at the end of the recording session to verify the lack of response potentials *post mortem*.

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, Germany) attached on a manipulator. The laser was pointed at the centre 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 ABR 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 ABR (both visually and above the signal-to-noise ratio of 2) at the maximal 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. 1D; 2A,B** and **Fig. S2A**).

Response latency and amplitude calculation: Response latency was calculated as the time difference between the first negative deflection of the 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 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 (***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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Additional information

Competing interests

The authors declare that no competing interests exist.

Author contributions

T.A., J.C.D., and C.P.H.E. conceptualized experiments; T.A. collected and analyzed the data for figures 1, 2, S1, and S2; T.A. and J.C.D. collected and analyzed the data for figure 3; 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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Supplemental figures

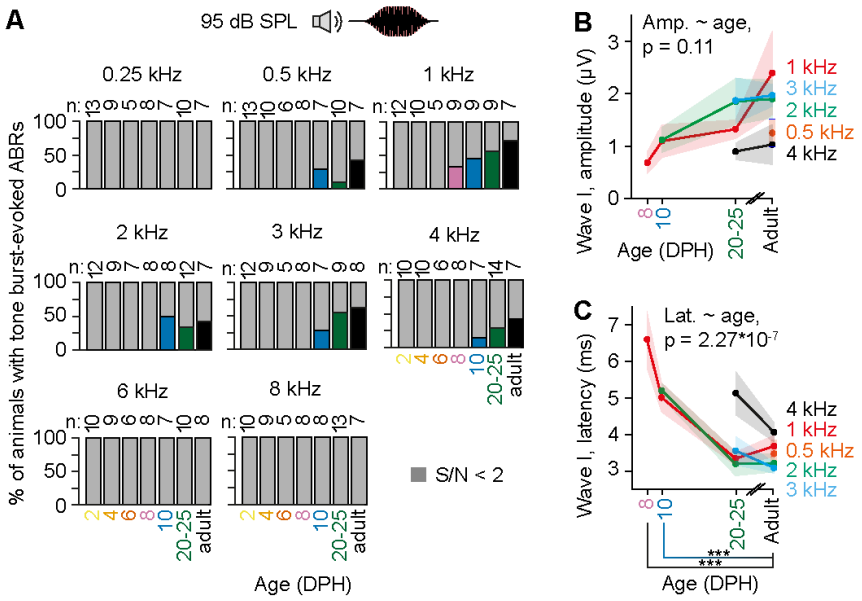

Figure S1. Development of tone burst-evoked auditory brainstem responses. (**A**) Percentage of animals displaying tone burst-evoked ABRs at 95 dB SPL. Gray bars indicate animals with S/N < 2. Exact number of animals is displayed above the bars. (**B**) Changes in tone burst-evoked ABR wave I amplitude and (**C**) latency over postnatal development. Frequencies are color coded as displayed below the figure. Shaded areas in B,C = s.e.m.

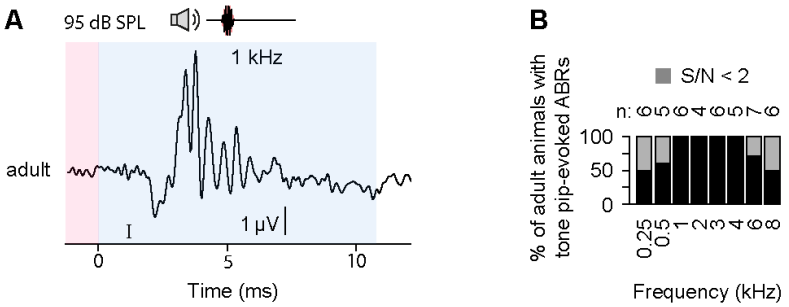

Figure S2. Tone pip-evoked auditory brain stem responses in adult zebra finches (**A**) Representative AC-ABR evoked by a 95 dB SPL, 1 kHz tone pip recorded from an adult zebra finch. (**B**) Percentage of adult zebra finches showing tone burst-evoked ABRs at 95 dB SPL between 0.25 and 8 kHz. Gray bars indicate responses with S/N < 2.

Supplemental tables

Table S1. Statistics for click-evoked auditory brainstem response thresholds

Click threshold: Model comparisons (Fig. 1G)

Model	Residual degrees of freedom	Residual sum of squares	Degrees of freedom	Sum of squares	F value	p value (F)
Null model	60	17345				
Age	54	4562	6	12784	25.2	4.93E-14

Click threshold: Coefficients (Fig. 1G)

Parameter	Parameter estimate	Standard error	t value	p value (t)
Intercept	54.6	1.2	45.3	1.162E-33
Age	-37.3	3.273	-11.4	5.320E-16

Table S2. Statistics for click-evoked auditory brainstem response thresholds

Click threshold: Model comparisons (Fig. 1G)

Model	Residual degrees of freedom	Residual sum of squares	Degrees of freedom	Sum of squares	F value	p value (F)
Null model	60	17345				
Age	54	4562	6	12784	25.2	4.93E-14

Click threshold: Coefficients (Fig. 1G)

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Intercept	54.6	1.2	45.3	1.162E-33
Age	-37.3	3.273	-11.4	5.320E-16

Click threshold: Post-hoc comparisons (Fig. 1G)

Contrast	Parameter estimate	Standard error	Degrees of freedom	t ratio	p value (t)
4 DPH vs. adult	37.500	4.596	54	8.160	3.268E-10
6 DPH vs. adult	29.375	4.964	54	5.918	1.385E-06
8 DPH vs. adult	14.375	4.271	54	3.366	0.008
10 DPH vs. adult	5.284	4.271	54	1.237	1
20 DPH vs. adult	-0.639	4.757	54	-0.134	1
25 DPH vs. adult	-5.625	4.360	54	-1.290	1

Table S3. Statistics for click-evoked auditory brainstem response amplitudes

Click amplitude: Model comparisons (Fig. 1H)

Model	Number of parameters	AIC	BIC	Log-likelihood	Deviance	Chi square	Degrees of freedom	p value (chi square)
Null model	3	2713	2726	-1353	2707			
Fixed effect: SPL	4	2277	2294	-1134	2269	438	1	2.779E-97
Fixed effects: SPL + Age	10	2209	2252	-1095	2189	79	6	4.687E-15
Interaction SPL * Age	16	2057	2125	-1013	2025	164	6	7.524E-33

Click amplitude: Coefficients (Fig. 1H)

Parameter	Estimate	Standard error	Degrees of freedom	t value	p value (t)
Intercept	-4.536	0.800	508.707	-5.670	2.402E-08
SPL	0.106	0.009	480.584	11.972	4.339E-29
Age	-5.626	2.796	508.128	-2.012	4.472E-02
SPL * Age	0.166	0.031	481.530	5.356	1.323E-07

Click amplitude: Post-hoc comparisons (Fig. 1H)

Contrast	Estimate	Standard error	Degrees of freedom	t ratio	p value (t)
4 DPH vs. adult	-6.750	1.250	154.026	-5.398	1.498E-06
6 DPH vs. adult	-6.109	1.082	70.689	-5.649	1.891E-06
8 DPH vs. adult	-5.409	0.859	652.494	-6.296	3.785E-07
10 DPH vs. adult	-4.723	60.854	51.324	-5.530	6.533E-06
20 DPH vs. adult	-2.850	0.951	51.361	-2.995	0.025
25 DPH vs. adult	-0.601	0.871	51.122	-0.690 1	

Table S4. Statistics for click-evoked auditory brainstem response latencies

Click latency: Model comparisons (Fig. 1I)

Model	**Number of parameters**	**AIC**	**BIC**	**Log-likelihood**	**Deviance**	**Chi square**	**Degrees of freedom**	**p value (chi square)**
Null model	3	833	846	-414	827			
Fixed effect: SPL	4	81	98	-36	73	754	1	4.808E-166
Fixed effects: SPL + age	10	-17	26	18	-36	109	6	2.763E-21
Interaction: SPL * age	16	-120	-52	76	-152	115	6	1.306E-22

Click latency: Coefficients (Fig. 1I)

Parameter	Estimate	Standard error	Degrees of freedom	t value	p value (t)
Intercept	6.911	0.108	365.521	64.005	1.111E-200
SPL -0.030	0.001	451.876	-28.302	4.037E-102	
Age -4.290	0.359	453.952	-11.964	7.319E-29	
SPL * Age	0.021	0.004	452.408	5.809	1.185E-08

Click latency: Post-hoc comparisons (Fig. 1I)

Contrast	Estimate	Standard error	Degrees of freedom	t ratio	p value (t)
4 DPH vs. adult	3.080	0.245	73.488	12.560	3.405E-19
6 DPH vs. adult	2.495	0.248	57.938	10.063	1.491E-13
8 DPH vs. adult	1.522	0.210	52.527	7.242	1.159E-08
10 DPH vs. adult	1.297	0.210	52.263	6.178	5.945E-07
20 DPH vs. adult	0.520	0.232	52.279	2.239	0.177
25 DPH vs. adult	0.357	0.214	52.221	1.668	0.608

Table S5. Statistics for tone burst-evoked auditory brainstem response amplitudes

Tone burst amplitude: Model comparisons (Fig. 2A and Fig. S1B)

Model	Number of parameters	AIC	BIC	Log-likelihood	Deviance	Chi square	Degrees of freedom	p value (chi square)
Null model	3	117.202	122.688	-55.601	111.202			
Fixed effect: Age	6	117.095	128.067	-52.547	105.095	6.107	3	0.107

Tone burst latency: Model comparisons (Fig. 2A and Fig. S1C)

Model	Number of parameters	AIC	BIC	Log-likelihood	Deviance	Chi square	Degrees of freedom	p value (chi square)
Null model	3	150.112	155.598	-72.056	144.112			
Fixed effect: Age	6	122.392	133.364	-55.196	110.392	33.720	3	2.270E-07

Tone burst latency: Coefficients (Fig. 2A and Fig. S1C)

Parameter	Estimate	Standard error	Degrees of freedom	t value	p value (t)
Intercept	4.744	0.159	42	29.826	6.790E-30
Age	-2.392	0.361	42	-6.631	4.920E-08

Tone burst latency: Post-hoc comparisons (Fig. 2A and Fig. S1C)

Contrast	Estimate	Standard error	Degrees of freedom	t ratio	p value (t)
8 DPH vs. adult	3.133	0.527	38.725	5.951	1.866E-06
10 DPH vs. adult	1.640	0.363	25.072	4.522	0.0004
20-to-25 DPH vs. adult	0.342	0.294	10.697	1.161	0.813

Tone burst threshold: Model comparisons (Fig. 2C)

Model	Number of parameters	AIC	BIC	Log-likelihood	Deviance	Chi square	Degrees of freedom	p value (chi square)
Null model	3	353.752	359.238	-173.88	347.752			
Fixed effect: Age	6	358.107	369.079	-173.05	346.107	1.645	3	0.649

Table S6. Statistics for comparing tone pip- vs tone burst-induced auditory brainstem responses

Tone pip vs tone burst: Model comparisons (Fig. 2C and Fig. S2)

Model	Number of parame-ters	AIC	BIC	Log-likelihood	Deviance	Chi square	Degrees of free-dom	p value (chi square)
Null model	3	477.716	483.683	-235.86	471.716			
Fixed effect: Stimulus type	4	469.444	477.400	-230.72	461.444	10.272	1	0.00135
Fixed effects: Stimulus type + Frequency	5	464.609	474.554	-227.31	454.609	6.835	1	0.00894

Tone pip vs tone burst: Coefficients (Fig. 2C and Fig. S2)

Parameter	Estimate	Standard error	Degrees of freedom	t value	p value (t)
Intercept	68.849	4.706	26.832	14.631	2.628E-14
Stimulus type	-19.651	4.878	50.590	-4.028	0.000189
Frequency	0.00311	0.00111	50.027	2.786	0.00751

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