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Evidence for hearing loss in amblyopsid cavefishes

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The constant darkness of caves and other subterranean habitats imposes sensory constraints that offer a unique opportunity to examine evolution of sensory modalities. Hearing in cavefishes has not been well explored, and here we show that cavefishes in the family Amblyopsidae are not only blind but have also lost a significant portion of their hearing range. Our results showed that cave and surface amblyopsids shared the same audiogram profile at low frequencies but only surface amblyopsids were able to hear frequencies higher than 800 Hz and up to 2 kHz. We measured ambient noise in aquatic cave and surface habitats and found high intensity peaks near 1 kHz for streams underground, suggesting no adaptive advantage in hearing in those frequencies. In addition, cave amblyopsids had lower hair cell densities compared with their surface relative. These traits may have evolved in response to the loud high-frequency background noise found in subterranean pools and streams. This study represents the first report of auditory regression in a subterranean organism.

1. Introduction

Animals that live in continual darkness are faced with unique challenges in order to locate and identify food, predators and each other [1]. Without visual information, independent lineages of obligate cave-dwelling organisms have evolved regressive features, such as the loss or reduction of eyes and pigmentation and constructive traits, such as longer appendages and hypertrophy of non-visual sensory systems [2]. Aside from darkness being common to all subterranean habitats, several other abiotic factors influence subterranean organisms, such as relatively stable temperature, high humidity and hydrological factors (for example, periodic flooding) [2]. However, little to nothing is known about how the diverse abiotic characteristics of caves affect the sensory ecology of cave animals. Here, we examine the relationship between the acoustic environment of caves and hearing in amblyopsid cavefishes.

Aquatic cave organisms, such as cavefishes, survive in perpetual darkness. An important sensory modality in such environments may be the sense of hearing. In above-ground aquatic habitats, hearing is important for many aspects of fish behaviour (reviewed in [3]) and is effective over relatively long distances owing to the nature of underwater sound travel. Sound may play an especially important role in subterranean habitats owing to the lack of visual signals yet the acoustic properties of these habitats have been largely ignored to date. Hypertrophy of hearing characteristics could be adaptive in caves for several reasons, including working in association with other non-visual senses to detect prey, conspecifics or predators. However, the degree to which hearing abilities are modified in cavefishes is largely unknown, as behavioural and neurophysiological studies on the acoustical biology of cavefishes are extremely limited. Popper [4] showed that the cave and surface forms of the characid *Astyanax mexicanus* do not differ in hearing. Similarly, no differences were found between cave and surface forms of the molly *Poecilia mexicana* [5].

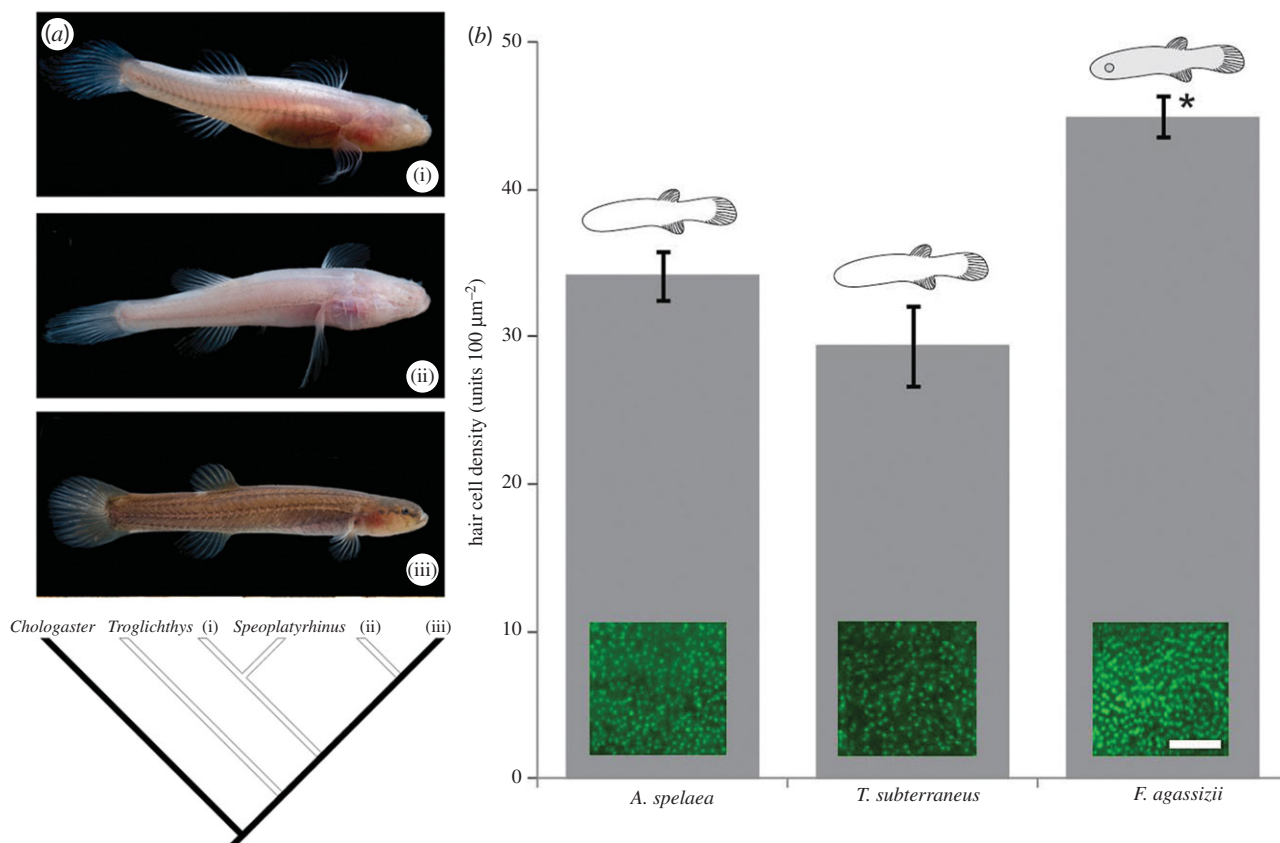


Figure 1. (a) The phylogenetic relationships of the two obligate cave species (white) (i) *Typhlichthys* and (ii) *Amblyopsis* and one surface species (black) (iii) *Forbesichthys*. (b) Cell density counts for the three species show fewer hair cells in the cavefishes ($*F_{2,23} = 15.3$, $p = 0.0007$). Inserts show photomicrograms of the ears stained with phalloidin. Scale bar, 100 μm . (Online version in colour.)

Here, we show the first report of differences in hearing characteristics in a cavefish compared with its surface relative. We compared the auditory evoked potentials (AEPs) of three species in the family Amblyopsidae, as well as the acoustic profiles of their subterranean habitats in order to investigate whether a relationship exists between noise in cave habitats and cavefish hearing. Amblyopsid caves are a model system for studying the ecological and evolutionary processes of cave adaptation because the cave-restricted species in the family represent a range of troglomorphy that reflects variable durations of isolation in caves [6]. Cave amblyopsids are one of the most comprehensively studied caves, with six genera and eight species [7]. In this study, we examine the hearing characteristics of three related amblyopsids: the surface dwelling, *Forbesichthys agassizii* and two cave species, *Typhlichthys subterraneus* and *Amblyopsis spelaea* (figure 1a).

2. Material and methods

All procedures followed IACUC guidelines dictated by the University of Windsor. All data are available in <http://datadryad.org> under doi:10.5061/dryad.9sj49 [8]. Fishes were collected under scientific permits issued by the states of TN (no. 1605) and KY (no. SC1211135), USA. We collected nine individuals of *Forbesichthys agassizii* from a quiet pool (10 m^2 , mean depth 0.6 m, mud/silt substrate with abundant vegetation) of a spring run fed by Jarrell's Spring, Coffee Co., TN, USA; seven individuals for each of the two cave-dwelling species: *Amblyopsis spelaea* from several quiet pools (20–150 m^2 , 0.2–2+ m depth, silt/sand/cobble substrate) in Under the Road Cave, Breckinridge Co., KY, USA and *Typhlichthys subterraneus* from several

poools with some current (4–12 m^2 , 0.1–0.8 m depth, 0–0.6 ms^{-1} (low flow), cobble/bedrock substrate) in L&N Railroad Cave, Barren Co., KY, USA.

(a) Auditory evoked potentials

This method measures the compound electrical potential created by the eighth cranial nerve and auditory brainstem nuclei in response to sound [9,10]. We restrained submerged fish and played 10 msec tones, ranging from 0.1 to 2 kHz at 0.1 Hz intervals. We increased the sound level in 5 dB intervals until a stereotypical evoked potential waveform was detected (figure 2, insert). We determined auditory threshold to be the lowest intensity for which AEP traces were detected [11]. Sound output was measured with a hydrophone (model LC-10, Reson Inc; Calibration sensitivity of -208.9 dB re 1V μPa^{-1} , 0–100 kHz) and an accelerometer (model 4524 cubic triaxial deltatron, Brüel & Kjær). We calibrated sound level and particle acceleration at the beginning of each trial. Thresholds were compared between species and frequencies with a two-way ANOVA.

(b) Hair cell histology

Fish were euthanized with an overdose of 2-phenoxy-ethanol and fixed in 4 per cent paraformaldehyde. Epithelia were dissected and stained with Oregon Green phalloidin (Invitrogen) followed by fluorescent imaging. Hair cells were manually counted across eight different regions of saccular epithelia and quantified as density (hair cells/2500 μm^2) to correct for differences in epithelium size. There were no apparent differences in fluorescent intensity sufficient to affect manual counts. Within species, there were no significant density differences between epithelial areas (ANOVA $F_{7,40} = 0.437$, $p = 0.873$), so the density estimates were averaged across epithelial areas. ANOVA was

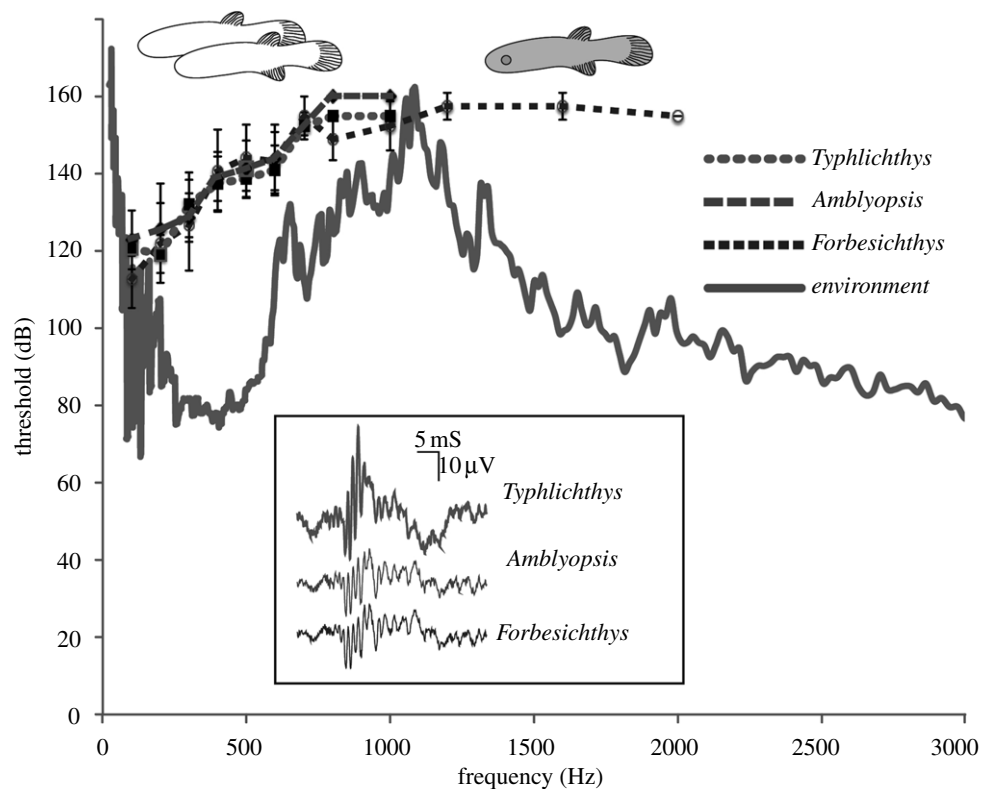


Figure 2. Auditory thresholds of amblyopsid fishes. Values are means \pm standard errors. The surface fish *Forbesichthys* reaches up to 2 kHz while the cavefish *Typhlichthys* (1) and *Amblyopsis* (2) are limited to 1 kHz. Fast Fourier Transformation (FFT) of sound recorded in a Drowned Rat Cave pool. The pool was carved in bedrock by a small stream. The recording was made 0.5 m deep and approximately 1 m from the waterfall. The ceiling of the cave was also dripping onto the pool. Insert: auditory evoked potential traces of all species to a 400 Hz tone burst at 60 dB.

used to assess differences in hair cell density, followed by a Tukey post-hoc test.

(c) Environmental sound profiles

We characterized aquatic environmental sound profiles in cave and surface habitats, using a hydrophone (type 10CT hydrophone, calibration sensitivity of -195 dB re. 1 V μPa^{-1} ; ± 3 dB, 0.02–10 kHz, omnidirectional, G.R.A.S., Denmark) connected through a preamplifier (Spikerbox, Backyard Brains) to an iPad (Apple). Three recordings of 5 min were taken per site. Within caves, we obtained sound profiles from two habitat types: shallow stream riffles at depths of 0.05–0.1 m and pools with no current at depths of 0.1–2 m. We also recorded at the same depths in surface streams and pools inhabited by *Forbesichthys*. Characterization of sound spectra and corresponding SPLs was performed using AUDIOTOOLS software (Studio Six Digital). We matched cave and surface habitats profiles as much as possible (e.g. area, substrate and water flow), with the exception of vegetation in surface habitats.

3. Results

Density of saccular hair cells differed between species ($F_{2,6} = 15.3$, $p = 0.0007$), with the two cave species having lower hair cell densities (mean = 34 and 29 hair cells/ $2500 \mu\text{m}^2$) than the surface species (mean = 45 hair cells/ $2500 \mu\text{m}^2$; figure 1). There was no difference in threshold between species below 800 Hz ($F_{2,15} = 1.087$, $p = 0.342$; figure 2), and thresholds increased with frequency ($F_{11,15} = 25.9$, $p < 0.001$) with no significant frequency–species interaction ($F_{15,95} = 47.9$, $p = 0.702$). All three amblyopsid species were most sensitive at 100 Hz (mean threshold range 112–122 dB re $1 \mu\text{Pa}$), and thresholds increased between 100 and 800 Hz.

In the two cave species, only one *Typhlichthys* responded to tones 700–1000 Hz and just two *Amblyopsis* responded to tone bursts above 600 Hz, with only one responding at 1000 Hz. The surface species showed clear evoked responses well above this limit, with defined responses detected up to 2000 Hz.

Underwater sounds were variable depending on habitat. In cave streams with rock and sand substrate, there was a peak in background noise at about 1000 Hz followed by peaks at low frequencies (below 200 Hz; figure 2). Overall sound intensity was less prominent between 200 and 5000 Hz in pool habitats away from the small streams. Nonetheless, the same general profile was present but with a smaller, less defined 1000 Hz peak. Surface streams showed low-frequency noise (less than 100 Hz) and high-frequency noise (more than 8000 Hz) with a small peak at 1200 Hz, but the overall noise level was much higher at intermediate frequencies (1000–3000 Hz) in the cave streams than surface streams.

4. Discussion

Adaptation to cave environments is often associated with hypertrophy of non-visual sensory modalities. Cave amblyopsids exhibited similar hearing sensitivities as their surface-dwelling relative at 800 Hz and below, consistent with previous findings in other cavefishes [5,6]. Surprisingly however, cave amblyopsids have lost a significant portion of their hearing range. Both *Amblyopsis* and *Typhlichthys* are unable to hear frequencies above 800 Hz, unlike their surface relative *Forbesichthys*, which can hear up to 2 kHz. In addition, both cave species had lower hair cell densities than *Forbesichthys*. To our knowledge, this is the first report of auditory regression in a subterranean organism.

Like the loss of eyes, loss of hearing range in cave amblyopsids represents an example of regressive evolution in subterranean organisms. Audio recordings from native cave habitats of cave amblyopsids showed that flowing streams (riffles) and water droplets dripping from the ceiling of cave passages contribute to loud high-frequency background noise generally above 800 Hz (figure 2), although the precise contribution of all noise sources have not been characterized. Lower frequencies are not likely to propagate far in these shallow environments [12] but the higher frequency components would propagate further and contribute to the more to the high background noise levels of the caves. The apparent match between hearing ability and background noise profiles has been hypothesized to be an evolutionary driver of hearing ability across the Teleostei [13], and the hearing of two species of goby (*Padogobius martensii* and *Gobius nigricans*) living in noisy waterfall environments is most sensitive in a frequency range corresponding to a quiet window in these environments [14]. Noisy stream environments mask high-frequency hearing in ostariophysan fishes [15] but hearing specializations of closely related species in different acoustic environments have rarely been tested. Our findings raise the intriguing possibility that cave amblyopsids may have lost hearing at high frequencies in response to the noisy acoustic environments in which they live.

The reduction in hair cell density indicates peripheral involvement in high-frequency hearing loss. Fewer hair cells provide fewer sites for signal transduction and also may lead to less relative stimulation upon relative motion of the otolith. Poulson [9] reports an increase in otolith size with increasing cave

adaptation in this group and suggests it may be due to different equilibrium demands. If the sensory epithelium is growing in pace with the otolith without concomitant increase in hair cells, a decrease in hair cell density would result. If, however, the loss of high-frequency hearing ability in cave species was due to selective loss of high-frequency hair cells, this could also lead to a decrease in overall hair cell density. There is no evidence for tonotopy in fish ears, but there is some evidence for differential frequency selectivity in hair cells across the epithelia [15]. More work needs to be done on frequency responses at the level of individual hair cells before this idea can be supported.

Our study provides evidence that two cavefish species have evolved loss of high-frequency hearing and reduced hair cell densities compared with a surface-dwelling relative. These traits may have evolved in response to loud high-frequency background noise that mask acoustic signals in their aquatic subterranean habitats; however, the mechanism (i.e. neutral loss versus selection) underlying hearing loss remain to be understood.

All procedures followed IACUC guidelines dictated by the University of Windsor. All data are available in <http://datadryad.org> under doi:10.5061/dryad.9sj49 [8]. Fishes were collected under scientific permits issued by the states of TN (no. 1605) and KY (no. SC1211135), USA.

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