Otolith morphology and hearing abilities in cave- and surface-dwelling ecotypes of the Atlantic molly, *Poecilia mexicana* (Teleostei: Poeciliidae)

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**A B S T R A C T**

Cave fish have rarely been investigated with regard to their inner ear morphology, hearing abilities, and acoustic communication. Based on a previous study that revealed morphological differences in the saccular otolith between a cave and two surface populations of *Poecilia mexicana*, we checked for additional differences in utricular and lagener otoliths and tested whether different populations have similar hearing sensitivities. We found pronounced differences in the shape of all three otoliths. Otoliths of the saccule and lagena from cave fish differed from those of surface fish in the features of the face oriented towards the sensory epithelium. In addition, otoliths of the utricle and lagena were significantly heavier in cave fish. Auditory sensitivities were measured between 100 and 1500 Hz, utilizing the auditory evoked potential recording technique. We found similar hearing abilities in cave and surface fish, with greatest sensitivity between 200 and 300 Hz. An acoustic survey revealed that neither ecotype produced species-specific sounds. Our data indicate that cave dwelling altered the otolith morphology in Atlantic mollies, probably due to metabolic differences. Different otolith morphology, however, did not affect general auditory sensitivity or acoustic behavior.

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1. Introduction

Unlike in most other vertebrates that possess numerous tiny otoconia in their inner ears, modern bony fishes (Teleostei) have a single massive calcareous concretion — the so-called otolith — in all three end organs. Fish otoliths are composed of calcium carbonate crystals suspended in a protein matrix. Calcium carbonate is usually deposited as aragonite in the otolith of the saccule (sagitta) and utricle (lapillus) and in a crystallized, less dense form termed vaterite in the otolith of the lagena (asteriscus) (Oliveira and Farina, 1996; Falini et al., 2005). These otoliths, especially the saccular otolith, show a (species-)specific morphology (Nolf, 1985). In examining the diversity of otolith morphologies and its implications for physiological functions in teleost fishes, one interesting question at the interface between otolith research and studies on inner ear physiology is whether and how otolith morphology may be related to inner ear physiology, such as hearing sensitivities (Popper et al., 2005; Popper and Schilt, 2008). Oxman et al. (2007), for example, reported that juvenile Chinook salmon (*Oncorhynchus tshawytscha*) with aberrant (vateritic) sagittae displayed significantly poorer auditory sensitivity than individuals with normally developed (aragonitic) sagittae. Ramcharitar et al. (2004) showed that the sciaenid species *Bairdiella chrysoura* has a unique otolith morphology, i.e. thick, large otoliths (asteriscus and lapillus) and a sagitta with a conspicuously deep sulcus acusticus which may be linked to better hearing abilities compared to other members of the family Sciaenidae. In a preceding comparative study (Schulz-Mirbach et al., 2008) on the sagittae of different ecotypes of the Atlantic molly (*Poecilia mexicana*), we found pronounced contour differences in cave- versus surface-dwelling populations. Moreover, sagittae of cave mollies often had a deep sulcus, lacking in surface-dwellers. We assumed that these differences might be related to hearing ability. In this study, we therefore examine whether otolith morphology reflects inner ear physiology (i.e., hearing sensitivities). Popper (1970)
found similar hearing sensitivities in the blind, cave-dwelling Mexican tetra *Astyanax mexicanus* (Characidae) and its surface-dwelling conspecifics, but did not investigate otolith morphology. We therefore tested whether (1) the cave form of *P. mexicana* and its surface-dwelling relatives have similar hearing sensitivities (as shown for the Mexican tetra) or (2) whether they have different hearing sensitivities that are reflected by the changed sagitta morphology.

Cave fishes are under strong selection pressure to develop and enhance non-visual communication channels (Burt de Perera, 2004; Montgomery et al., 2001; Parzefall, 1970, 2001). To compensate for the lack of visual input, they have evolved several modifications of their sensory systems such as a well-developed lateral line system (Burt de Perera, 2004; Montgomery et al., 2001) or improved the senses of taste and touch (Parzefall, 1970, 2001). A potentially altered sense of hearing and acoustic communication has received little attention. No data are available on these aspects in poeciliids in general. We therefore studied cave and surface populations of *P. mexicanum* with respect to hearing abilities and acoustical signaling. Cave and surface populations of the Atlantic molly differ in their sagitta morphology, making them perfect model organisms to investigate the potential relationship between otolith morphology and hearing sensitivities.

The present study focuses on three aspects. First, we tested for potential differences between the two ecotypes in the morphology of all three otolith types (asterisci, sagittae, lapilli), with special emphasis on details of the sulcus region of the sagittae and on the potential differences between the two ecotypes in the morphology of asterisci and lapilli. Second, we tested whether cave and surface-dwelling fish show similar hearing sensitivities, or whether the former display changed hearing sensitivities as an adaptation to perpetual darkness. Third, we conducted an acoustic survey to determine whether *P. mexicana* communicates acoustically.

2. Materials and methods

2.1. Study system and animals

The Atlantic molly, *P. mexicana* Steindachner, 1863 (Poeciliidae) is widespread in freshwater surface habitats along the Atlantic versant of Central America (Miller, 2005). The cave form of *P. mexicana* inhabits the Cueva del Azufre cave system, which is divided into 13 interconnected cave chambers (Gordon and Rosen, 1962). A creek flows through the cave, forming several shallow pools that are partially divided by riffle passages. While the front cave chambers receive some dim light, the inner parts of the cave are lightless, and the molly population from the innermost cave chamber XIII (Gordon and Rosen, 1962) permanently lives in the dark. With the exception of chamber XIII, the water in all cave chambers is characterized by medium to high concentrations (up to 300 µM/L) of naturally occurring hydrogen sulfide (Tobler et al., 2006, 2009).

### 2.1.1. Otolith morphology and hearing sensitivity

A total of 46 fish from the cave form and two surface populations of *P. mexicana* were investigated with regard to their otolith morphology and hearing sensitivity (Table 1). One of the surface populations originated from the Río Oxlólica, a river with sulfide-free water near the cave (Tobler et al., 2006). The second population of surface-dwelling fish came from brackish coastal waters near Tampico (Tamaulipas, eastern Mexico). Large, randomly outbred stocks of the cave population and from Río Oxlólica have been maintained in 200-l aquariums at the University of Potsdam since 2004; stocks from Tampico were founded using wild-caught fish in 1995. Fish were transferred to the University of Vienna in December 2008 and February 2009 for the auditory analyses. They were kept in 120 to 160-l aquariums, which were equipped with a sand bottom, halved flower pots as hiding places, and external filters. No internal filters or air stones were used in order to create a quiet acoustic environment for the test fish. Fish were kept under a 12:12 h L:D cycle at 25 ± 1 °C and were fed once daily with commercial flake food. The conditions were comparable to those at the University of Potsdam. Fish were given a habituation period of two to three days prior to the auditory experiments. All hearing experiments were performed with the permission of the Austrian Federal Ministry of Science and Research (GZ 66.006/0023-II/10b/2008).

### 2.1.2. Acoustic survey

Cave fish for the survey of the acoustical behavior originated from chamber V of the Cueva del Azufre (Gordon and Rosen, 1962), and surface fish came from the Río Amatán, another sulfide-free river that merges with the Río Oxlólica downstream, but close to the cave (Tobler et al., 2008). Both populations were a mix of wild-caught and first generation laboratory-reared fish, originally collected and established in January 2009. Fish were maintained at the University of Oklahoma under 12:12 h L:D cycles, kept in 160-l tanks at 26 °C with gravel bottom and internal filters, and were fed once daily with either commercially available flake food or mosquito larvae (bloodworms).

### 2.2. Otolith dissection, otolith measurements and shape analysis

Following the measurements of hearing sensitivities, the standard length of the fish was measured to the nearest millimeter and animals were decapitated. Sex was determined by inspection of the gonopodium (transformed anal fin) of the males and by dissection of the ovary of the females. The three otolith types — lapillus, sagitta, and asteriscus — were dissected from the left membranous labyrinth, cleaned of organic residues with 1% potassium hydroxide solution for 4–6 h and rinsed several times in distilled water. After cleaning, otoliths were stored dry at room temperature in small plastic cells (Krantz2-cells). Fresh otoliths showed the same morphological features, e.g. development of the sulcus center in *P. mexicana* and *P. mexicana* with respect to hearing abilities and acoustical sensitivity.

### Table 1

Populations, numbers of specimens, and size ranges of *Poecilia mexicana* used for auditory measurements and otolith analyses. BW, body weight; f, female; m, male; N, number of specimens; SL, standard length.

| Population     | Auditory measurements | Otolith analyses |
|----------------|-----------------------|-----------------|
|                | N [f/m]               | SL (mm)         | BW (g) | N [f/m] | SL (mm) |
| Cueva del Azufre | 13 [8/5]              | f: 35–43;       | f: 0.8–1.2; | 19 [12/7] | f: 35–55; |
|                |                       | m: 33–40        | m: 0.7–1.0 |           | m: 32–40 |
| Tampico        | 14 [11/3]             | f: 30–52;       | f: 0.6–2.9; | 20 [15/5] | f: 30–54; |
|                |                       | m: 28–32        | m: 0.4–0.8 |           | m: 28–34 |
| Río Oxlólica   | 3 [2/1]               | f: 35;          | f: 0.8–1.1; | 3 [2/1]  | f: 35;   |
|                |                       | m: 44           | m: 2.4     |           | m: 44    |

The first numeral given in brackets indicates the number of females; the second numeral represents the number of males.

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weight was determined to the nearest 0.01 mg with a Mettler-Toledo AT21 (±0.001 mg, s.d.). To determine otolith area and otolith contour, the macula-oriented side of all otoliths was investigated using a scanning electron microscope (a LEO VP 1430 at the Zoological State Collection in Munich, Germany) at a magnification of 45- to 58-fold (sagittae), 95- to 160-fold (asterisci), and 140- to 220-fold (lapilli). For SEM investigation, otoliths were mounted on aluminum stubs and sputter coated with gold. These SEM images were converted into images containing a white object on black background in Adobe® Photoshop CS2. Afterwards, otolith area was quantified in tpsDig2 (Rohlf, 2004). Otolith contours were saved as raw (x/y)-coordinates in tpsDig2 and subjected to the shape analysis software Hshape (Crampton and Haines, 1996; Haines and Crampton, 2000) consisting of the three programs Hangle, Hmatch, and Hcurve. The output of Hangle and Hmatch are Fourier descriptors (FDs), describing the contour by combination of sine- and cosine-waves. Normalization of size was performed automatically in Hangle (29 smoothing iterations), whereas

Fig. 1. Scanning electron microscopic (SEM) images of the sulcus region of left sagittae from (A)–(E) a cave fish (male; SL = 34 mm) and F–I an individual from the Tampico surface population (male; SL = 33 mm). (B)–(E) are details of (A), and (G)–(I) show details of (F). (B)–(C), (G) display details of the crista superior, (D) and (H) from the center of the sulcus, and (E)–(I) from the area beneath the crista inferior. Both sagittae display an impression in the upper caudal part of the sulcus acusticus (white arrowheads). For further descriptions see also Table 2. Scale bars, 30 μm in (A), 2 μm in (B)–(C), (I), and 10 μm in (D)–(E), (G)–(H).
normalization of rotation and starting point was ensured by applying Hmatch. Twenty Fds were used to analyze contours of lapilli and 32 Fds for sagitae and asterisci, respectively. In order to visualize differences in otolith contours between the populations, averaged Fds of each population and otolith type were back-calculated into (x/y)-coordinates (1024 per contour) applying Hcurve.

In some hatchery-reared fish stocks, aberrant otoliths are more abundant than in wild animals (e.g., Oxman et al., 2007; Sweeting et al., 2004). Thus, in order to test for possible artifacts of lab conditions, we screened our samples for the relative abundance of aberrant otoliths and also compared otolith morphology of lab-reared animals with that of wild fish studied in Schulz-Mirbach et al. (2008).

2.3. Auditory sensitivity measurements

Auditory thresholds were determined by applying the auditory evoked potential (AEP) recording technique following the protocol developed by Kenyon et al. (1998) and modified by Wysocki and Ladich (2005a, b). The AEP technique records far-field potentials in response to sound stimuli of the whole auditory pathway from the inner ear up to midbrain nuclei (Corwin, 1981). For a comparison between AEP and behavioral thresholds, and for AEP thresholds gained in different laboratories, see Kenyon et al. (1998) and Ladich and Wysocki (2009).

Prior to each auditory experiment, individuals were chosen randomly from one of the three populations. Depending on body weight, the test subject was immobilized with Flaxedil (gallamine triethiodide; Sigma Aldrich Handels GmbH, Vienna, Austria) at a concentration of 9.5–49.3 μg g⁻¹ body weight in order to reduce muscle noise. All auditory measurements were carried out in a bowl-shaped plastic tub (diameter 33 cm, water depth 13 cm, 1 cm layer of sand), which was lined inside with acoustically absorbent material (air-filled packing wrap) to minimize resonances and reflections. For a more detailed description see Wysocki and Ladich (2002; Fig. 1B, C therein). The tub was positioned on an air table (TMC Micro-g 63-540, Technical Manufacturing Corporation, Peabody, MA, USA), which rested on a vibration-isolated plate of concrete. A sound-proof chamber, constructed as a Faraday cage (interior dimensions: 3.2 m × 3.2 m × 2.4 m), enclosed the whole set-up. Test subjects were positioned in the center of the tub, so that the nape of the head was just above the water surface (<1 mm). For respiration a pipette was inserted into the fish’s mouth and respiration was facilitated by a simple, temperature-controlled (25 ± 1 °C), gravity-fed water circulation system. The area of the head above the water surface was covered with a small piece of Kimwipes® tissue paper to keep it moist. Silver wire electrodes (diameter 0.38 mm) were used for recording AEPs. The recording electrode was placed in the midline of the skull over the region of the medulla, the reference electrode cranially between the nares. Both electrodes were pressed firmly against the skin. Tone-bursts were presented through two speakers (Fostex PM-0.5 Sub and PM-0.5 MKII, Fostex Corporation, Tokyo, Japan) positioned 50 cm above the animal’s head. The AEP waveform recording was performed by a modular rack-mount system [Tucker-Davis Technologies (TDT) System 3, Gainesville, FL, USA] containing a TDT digital signal processing board and running TDT BioSig RP software. Auditory thresholds were evaluated in random order for 100, 200, 300, 500, 1000, 1500, and 2000 Hz, respectively, by decreasing the sound pressure level in 4-dB steps. Several individuals were tested at 3000, 4000, and 5000 Hz, but none of them showed any response beyond 2000 Hz.

2.4. Sound pressure level and particle acceleration measurements

P. mexicana is not known to possess any hearing specializations (hearing non-specialists or generalists; see also Popper and Fay, in press). Therefore, they primarily detect the particle component of sound. Audiograms are often expressed in terms of sound pressure levels, which may not be adequate for non-specialists (Wysocki et al., 2009). We therefore included measurements of particle motion (accelerations) to overcome this problem.

A hydrophone (Brüel & Kjaer 8101, Naerum, Denmark; frequency range: 1 Hz—80 kHz ± 2 dB; voltage sensitivity: –184 re 1 V/μPa) was placed on the right side of the animals (~1 cm away) in order to control for absolute stimulus SPLs underwater in close proximity to the subjects during each experimental session. In order to compare sound pressure and particle acceleration levels for AEP thresholds, a calibrated underwater miniature acoustic pressure—acceleration (p—a) sensor (S/N 2007-001, Applied Physical Sciences Corp., Groton, CT, USA) was placed at the fish’s position in the test tub. This p—a sensor (with a frequency bandwidth from 20 Hz to 2 kHz) allows simultaneous recording of sound pressure and particle acceleration. It consists of two built-in units: a piezoelectric, omni-directional hydrophone (sensitivity: –173.7 dB re 1 V/μPa) and a bi-directional accelerometer (sensitivity: –137.6 dB re 1 V/μm/s²). Measurements of all stimulus frequencies at various levels, including the hearing threshold levels of the fish, were measured with the acceleration sensor subsequently oriented in all three orthogonal directions. In consistence with previous studies (Casper and Mann, 2006; Horodysky et al., 2008; Wysocki et al., 2009), the x-axis was considered to be anterior—posterior along each subject’s body, the y-axis was considered to be lateral (right—left) relative to the subject, and the z-axis to be vertical (i.e., up-down) relative to the subject. This approach yielded simultaneous measurements of sound pressure and particle acceleration in all three directions over the entire stimulus range. Sound pressure levels (SPL) were calculated in dB RMS re 1 μPa and particle acceleration levels (La) in dB RMS re 1 μm/s². These are the international units for sound pressure and particle acceleration according to ISO standards (ISO 1683, 1983).

2.5. Acoustic survey

Sound recordings were obtained from a variety of different laboratory settings in either 40-l or 70-l rectangular aquaria. Again, water temperatures were maintained at 26 °C. We used three different settings: (1) all-male, (2) all-female, and (3) mixed-sex tanks, and recordings were made in both light and darkness. Each

### Table 2

Comparison between the different features of the sulcus region of the sagittae from cave- and surface-dwelling fish (see also Fig. 1).

| Sulcus region, Population | Area above crista superior (Fig. 1A, F–G) | Crista superior (Fig. 1B, C, G) | Sulcus center (Fig. 1D, H) | Caudal sulcus impression (Fig. 1A, F) | Area beneath crista inferior (Fig. 1E, I) |
|--------------------------|------------------------------------------|-------------------------------|---------------------------|----------------------------------|-------------------------------------|
| Cueva del Azufre and Río Oxolotán | Almost smooth with large knobs | With pits of different size in the anterior part, crenate ventrally | Crystals of varying size and shape Rod-shaped crystals arranged in a circular pattern | Present in 67% of the males and 25% of the females Present in 100% of the specimens | With small protrusions and pits differing in size Protrusions or knob-like structures, never with pits |
Fig. 2. SEM images of the central part of the crista medial of left asterisci using the nomenclature according to Assis (2003) from (A) a cave fish (male; SL = 35 mm) and (B) a surface-dwelling specimen from Tampico (male; SL = 32 mm). The crista medial is narrower and slightly more bulging in the asteriscus of the cave fish than in that of the surface-dwelling fish. Scale bars, 10 μm in (A) and (B).

Fig. 3. SEM images of asterisci, sagittae, and lapilli from a cave fish (female; SL = 38 mm), one specimen from Tampico (female; SL = 41 mm), and a fish from Río Oxolotán (female; SL = 44 mm). Shape is distinctly different in asterisci between the cave fish and fish from both surface populations. Scale bars, 100 μm. c, caudal; d, dorsal; l, lateral; m, medial; r, rostral; v, ventral.
setting consisted of approximately 12 fish (all-male: 10–12 males; all-female: 12 females; mixed-sex: 6 males and 6 females), and fish were acclimatized for at least 2 h in the test tanks prior to recording sessions. Finally, each setting was replicated with *P. mexicana* from a surface stream (Río Amatan) and the Cueva del Azufre (cave chamber V). Due to physical, physiological, and morphological constraints on teleost sound-producing (sonic) organs, most fish sounds are pulsed signals ranging in frequency from around 60 Hz to more than 1 kHz (Amorim, 2006). Congruently, recent sound analyses in *Cyprinodon* spp. (Cyprinodontidae), like poeciliids members of the order Cyprinodontiformes found the dominant frequency of calls ranging from 400 Hz to 1500 Hz (Johnson, 2000; Nicoletto and Linscomb, 2008). In the majority of fishes investigated so far, main energies of sounds are found in the frequency range of highest auditory sensitivity (Wysoczki, 2006). This led us to expect the main energies of potential acoustic signals of *P. mexicana* to be found below 500 Hz. In order to detect low-level sounds and because test tanks were properly aerated prior to testing, filters and aeration were switched off and removed for the duration of the acoustic survey in order to create a quiet acoustic environment.

The survey was conducted using the SQ26-MT underwater recording system, which consists of a Sensor Technology SQ26-08 hydrophone (Cetacean Research Technology, Seattle, WA) and an M-Audio MicroTrack II solid state digital recorder (M-Audio, Irwindale, CA). Recordings were digitized and analyzed using Raven version 1.2.1 (Cornell Laboratory of Ornithology, Ithaca, NY), and spectrograms were generated using a Hanning window, a fast Fourier transformation size of 4096 samples, a filter bandwidth of 124 Hz, and 75% overlap. To allow for a correlation of acoustics with the behavioral context, tanks in light were filmed during acoustic recording sessions using a Canon DM-XL1A professional camcorder (Canon Inc., Tokyo, Japan).

### Table 3

| Population   | Otolith weight (mg) | Otolith area (mm²) |
|--------------|---------------------|--------------------|
|              | Lapilli             | Sagittae           | Asterisci         |
| Cave         | 0.06 ± 0.006        | 0.98 ± 0.123       | 0.11 ± 0.013      |
| Tampico      | 0.05 ± 0.005        | 0.85 ± 0.092       | 0.09 ± 0.009      |
| Río Oxolotán | 0.05 ± 0.006        | 1.08 ± 0.236       | 0.10 ± 0.021      |

For post hoc comparisons concerning differences in hearing sensitivities (SPL) related to body size (standard length), we used multiple *t*-tests while comparing fish smaller than and larger than the mean body size for each frequency, separately. Alpha levels were corrected for multiple comparisons using the Bonferroni correction (Rice, 1989).

### 3. Results

#### 3.1. Population differences in otolith morphology

**3.1.1. Structural features of the otoliths’ macula-oriented faces**

The most striking difference between cave- and surface-dwelling fish regarding the structure of the sagittae was in the sulcus acusticus region. Divergent features in this region are (1) the structure of the crista superior, (2) the surface structure of the area above the crista superior, (3) the arrangement of crystals in the center of the sulcus, and (4) the presence or absence of pits on the area beneath the crista inferior (Fig. 1, Table 2). The crista superior...
The sagittae of cave fish steeply declines towards the sulcus, whereas in surface fish it gradually merges into the sulcus (Fig. 1A, F). Moreover, the anterior part of the crista superior in cave fish sagittae displays several small to medium-sized pits (Fig. 1C) which are absent in surface fish. The sagitta of surface fish bear almost equally sized, rod-shaped crystals arranged in a circular pattern in the sulcus center; sagittae of cave fish lack this pattern and have crystals of varying size (Fig. 1D vs. H). In addition, sagittae of cave fish show medium- to large-sized pits in the area beneath the crista inferior (absent in sagittae of surface fish; Fig. 1E vs. I). Minor differences between the otoliths of cave- and surface-dwelling fish were also found in the fossa acustica region of the asterisci. The crista medial of the asterisci is commonly more bulging and narrower in the former than in the latter (Fig. 2). In contrast, the macula-oriented face of the lapilli is more variable within than among the examined populations, i.e., no distinction based on habitat type can be made. The development of this part of the lapilli ranges from almost smooth to displaying large bundles of crystals (Fig. 3, right).

### 3.1.2. Otolith measurements

Asterisci and lapilli of cave fish were significantly heavier at comparable area values (i.e., denser) than those of surface fish \( (F_{2,35} = 4.49; P = 0.018) \) (Tables 3 and 4; Fig. 4A, C). Sagittae showed a significant effect of the factor ‘sex’ \( (F_{1,35} = 9.707; P = 0.004) \) because males had heavier (i.e., denser) sagittae than females (Fig. 5).

### 3.1.3. Shape differences

The contours of asterisci \( (F_{8,64} = 6.223; P = 0.005) \) and lapilli \( (F_{8,64} = 4.474; P = 0.015) \) differed significantly among populations (Table 5; Fig. 6, right). By contrast, mean shape differences were

![Fig. 4. Ln-transformed otolith weight versus ln-transformed otolith area of A asterisci, B sagittae, and C lapilli from the cave fish (N = 19) and surface-dwelling fish from Tampico (N = 20) and Río Oxolotán (N = 3). Otolith weight differed significantly between populations for asterisci and lapilli (\( F_{2,35} \geq 4.49; P \leq 0.018 \)) (see also Table 4).](image)

![Fig. 5. Ln-transformed otolith weight versus ln-transformed otolith area of sagittae of male (N = 13) and female fish (N = 29). As ‘population’ had no significant effect on sagitta weight \( (F_{2,35} = 1.948; P = 0.158) \), individuals of the populations were pooled. Otolith weight differed significantly between sexes \( (F_{1,35} = 9.707; P = 0.004) \) (see also Table 4).](image)

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Table 5

| Source       | Wilk’s \( \lambda \) | F     | Hypothesis df | Error df | P    |
|--------------|----------------------|-------|---------------|----------|------|
| Asterisci    |                      |       |               |          |      |
| SL           | 0.157                | 0.671 | 32            | 4        | 0.772|
| Sex          | 0.054                | 2.191 | 32            | 4        | 0.233|
| Population   | <0.0001              | 6.223 | 64            | 8        | 0.005|
| Sex × population | 0.013               | 0.992 | 64            | 8        | 0.561|
| Sagittae     |                      |       |               |          |      |
| SL           | 0.025                | 4.783 | 32            | 4        | 0.069|
| Sex          | 0.106                | 1.049 | 32            | 4        | 0.554|
| Population   | 0.003                | 2.287 | 64            | 8        | 0.106|
| Sex × population | 0.002               | 2.847 | 64            | 8        | 0.058|
| Lapilli      |                      |       |               |          |      |
| SL           | 0.053                | 2.218 | 32            | 4        | 0.229|
| Sex          | 0.177                | 0.582 | 32            | 4        | 0.830|
| Population   | 0.001                | 4.474 | 64            | 8        | 0.015|
| Sex × population | 0.027               | 0.640 | 64            | 8        | 0.846|
only weak in the case of sagittae, and no statistically significant difference among populations was detected \( (F_{8,64} = 2.287; P = 0.106; \text{Table 5}) \). Furthermore, neither standard length nor sex significantly affected the contours of the three otolith types \( (P \geq 0.069; \text{Table 5}) \).

The first two principal components (PCs) were relevant according to the ‘broken stick model’ for the contours of asterisci. For the contours of sagittae, the first six PCs, and for the contours of lapilli the first three PCs, explained more variance than expected by chance. The PCs accounted for 45% (asterisci), 74% (sagittae), and 55% (lapilli) of the overall variance. The 2D plots of the PCA (PC1 versus PC2) revealed a separation of asterisci and sagittae contours in cave versus Tampico fish (Fig. 6, left) especially along PC1 for asterisci and along PC2 for sagittae. The principal component plot of lapilli contours showed less separation of the populations than those of asterisci and sagittae. The contours of asterisci and sagittae

![Fig. 6. Plots of principal component analyses (PC1 versus PC2) of the contours of asterisci, sagittae, and lapilli. The PCAs were based on 20 Fourier descriptors for contours of the lapilli, and 32 Fourier descriptors for the contours of the asterisci and sagittae, respectively. For specimens from the cave and Tampico, 95% probability ellipses are shown; in the case of the Río Oxolotán specimens, convex hulls are overlaid due to the small sample size. Mean shapes of asterisci, sagittae, and lapilli display parts of the contours that differ between cave fish \( (N = 19) \), specimens from Tampico \( (N = 20) \), and those from Río Oxolotán \( (N = 3) \).](image-url)
from the three Río Oxolotán specimens plot into the 95% probability ellipses of those from the Tampico individuals, indicating similarity among fish from surface populations. The lapilli contours of the Río Oxolotán individuals, however, fell into the 95% probability ellipses of both the cave and Tampico specimens.

The discriminant function analysis based on asterisci contours classified 92.9% of individuals to the correct habitat type ‘cave’ or ‘surface’ (Tampico and Río Oxolotán specimens merged); 97.6% correct classification were obtained for sagittae contours, 88.1% for lapilli contours (Table 6). In general, the analyses revealed that the contours of the three otolith types differed markedly between ecotypes (cave fish versus fish from the surface populations).

3.2. Comparison of otolith morphology between lab-reared and wild-caught mollies

Two out of 41 sagittae (4.9%) from the lab-reared fish showed an aberrant morphology, whereas all asterisci and lapilli were normally developed. None of the wild-caught individuals (N = 67) had aberrant otoliths.

Sagittae of wild-caught specimens from the cave system (including individuals from chamber XIII and several surface populations near the cave system) showed the same differences in the macula-oriented side as the lab-reared animals. The circular pattern of rod-shaped crystals in the sulcus center in specimens from the surface populations Tampico and Río Oxolotán (Fig. 3, middle) was also identified in wild-caught individuals from the surface-dwelling population Arroyo Cristal (Schulz-Mirbach et al. 2008, Fig. 3A3 therein).

The differences in the asterisci of lab-reared individuals were less pronounced than those in wild-caught specimens, i.e. asteriscus shape in some cave fish was similar to that of surface fish. Nevertheless, wild-caught cave fish also exhibited a trend towards more compact asterisci with a bulging crista medially and occasionally a deep fossa acustica. Lapilli of wild-caught fish showed a similar variability within populations as those from laboratory-reared specimens and tended to be thickened in some wild-caught cave fish.

3.3. Comparison of hearing sensitivities between cave- and surface-dwelling fish

The sound pressure level (SPL) audiograms of the three populations of P. mexicana showed a similar shape (Fig. 7A; Table 7) and, overall, mean thresholds were not significantly different (Table 8). Also, no significant interaction effect of ‘frequency by population’ was found, showing that populations did not differ in their specific response to different frequencies. In general, fish were most sensitive at 200 and 300 Hz, whereas thresholds increased abruptly above 300 Hz (Fig. 7A). Fish in all experiments responded to frequencies of up to 1500 Hz, and most also responded at 2000 Hz. Because not all individuals responded at 2000 Hz, this frequency was excluded from the statistical analyses. The particle acceleration level audiograms of the populations were similar in shape displaying lowest thresholds at 200 and 300 Hz (Fig. 7B; Table 7), and the populations showed no significant differences in their mean thresholds (Table 8).

Table 6
Jackknifed classification matrix of the discriminant function analysis (DFA) between cave fish and fish from the surface populations (Tampico and Río Oxolotán). Principal components (PC) from principal component analyses of the Fourier descriptors served as input variables of the DFA (asterisci: PC1–2; sagittae: PC1–6; lapilli: PC1–3). Significant $p$-values are bold.

| Otolith type | Wilk’s $\lambda$ | $\chi^2$ | $p$ | Population | Cave | Surface pop. |
|--------------|-----------------|----------|-----|------------|------|-----------|
| Asterisci    | 0.218           | 59.328   | <0.001 | Cave       | 89.5 (17) | 16.5 (2) |
|              |                 |          |      | Surface pop. | 4.3 (1) | 95.7 (22) |
| Sagittae     | 0.162           | 67.264   | <0.001 | Cave       | 94.7 (18) | 5.3 (1)  |
|              |                 |          |      | Surface pop. | 0 (0)  | 100 (23)  |
| Lapilli      | 0.405           | 34.832   | <0.001 | Cave       | 89.5 (17) | 10.5 (2) |
|              |                 |          |      | Surface pop. | 13 (3)  | 87 (20)   |

In the right part of the table, percentages in rows represent the classification into the populations given in columns; the corresponding number of specimens is given in parentheses. The percentages of correctly classified individuals are shown in bold.

Fig. 7. Audiograms based on the auditory evoked potential recording technique of fish from the cave (N = 13) and the surface populations Tampico (N = 14) and Río Oxolotán (N = 3). A Sound pressure level and B particle acceleration level audiograms. C Mean hearing thresholds (±s.e.m.) between fish with SL ≥ 36.5 mm (N = 19) and SL ≤ 36.5 mm (N = 11), depicting an effect of standard length by frequency. Because mean thresholds within the respective frequencies did not differ significantly between populations, data from the three populations were pooled. *Statistically significant according to a t-test (t28 = −3.499; $p = 0.002$).
A significant effect of ‘frequency by standard length’ (P = 0.014; Table 8) indicated that larger fish had slightly lower response auditory thresholds than smaller specimens at the highest frequency, namely at 1500 Hz (t_{28} = -3.499; P = 0.002) (Fig. 7C). No significant differences between sexes and no significant interaction terms involving sex were found (Table 8).

3.4. Acoustic survey

Altogether, we surveyed more than 30 h of acoustic recordings for all different settings and populations. Video analysis revealed that during settings in light, *P. mexicana* from both populations exhibited the full range of typical behaviors (e.g., male–male aggression or male–female mating). Nonetheless, we found no evidence for sound production and, thus, acoustic communication in any population of *P. mexicana*.

4. Discussion

Our study is the first to compare a cave fish with its surface-dwelling relatives with respect to otolith morphology, hearing sensitivities, and the possible role of acoustic communication. Cave mollies differed distinctly from surface-dwelling conspecífics in the morphology of asterisci, sagittae, and lapilli, but they exhibited similar hearing sensitivities. Both ecotypes appeared to be non-vocal.

4.1. Is otolith morphology affected by lab conditions?

Discussing and explaining differences in otolith morphology first requires examining possible effects of rearing conditions. As cave fish were light-reared, otolith morphology might have been affected to a certain degree by lab conditions. However, the relative abundance of abberant otoliths in the lab-reared individuals was within the range reported for several fish species caught in the wild (0.4–14%; Gauldie, 1993) and lower than the 7.8–13.9% for lab-reared herring (*Clupea harengus*; Tomas and Geffen, 2003). In addition, otolith differences between lab-reared cave and surface fish were similar to those in wild-caught specimens. Thus, we conclude that otolith contours and features of the macula-oriented side of all three otolith types were at best marginally affected by rearing conditions.

4.2. Otolith morphology and inner ear physiology

We found distinct differences between cave and surface fish in all three otolith types, whereas both ecotypes had similar hearing sensitivities. These similar sensitivities in *P. mexicana* agree with results on the Mexican tetra (*Astyanax mexicanus*; Popper, 1970). On the one hand, altered otolith morphology in cave fish might point to modified functions of the inner ear such as for detection of linear acceleration, sound source localization, or the vestibular sense.

Littler, however, is known about the relationships between otolith features and physiological functions, hampering interpretations (especially concerning otolith contours). Nonetheless, different contours of all three otolith types between cave- and surface-dwellers might point to a modified stimulation of the sensory epithelia. Popper et al. (2005) and Popper and Schilt (2008) noted that different contours might result in different centers of gravity of otoliths, potentially affecting the motion of the otolith relative to the sensory epithelium. In addition, Lychakov and Rebane (1992) hypothesized — based on a mathematical analysis of otolith vibrations in an acoustic field — that otolith shape may be involved in the ability to perceive directional sound.

The differences in the surface structures of the asterisci and sagitta’s macula-oriented sides in cave fish might change the attachment of the otolithic membrane. This, in turn, may result in a different mechanical transduction of shearing forces of the asteriscus or sagitta motion mediated via the otolithic membrane to the sensory epithelium. Preliminary studies on histological sections of saccules from cave mollies show that the deep sulcus in some individuals is filled with a distinctly thickened otolithic membrane, whereas in sagittae with a flat sulcus the otolithic membrane consists of only a very thin layer (T. Schulz-Mirbach, unpublished).

Assuming that saccule and sagitta play a key role in the acoustic sense (Popper and Lu, 2000), the similarity in sagitta weight of cave- and surface-dwellers might be related to the similar hearing sensitivities. A recent study suggested that differences in the weight-to-area ratio and density of vateritic and aragonitic sagittae of *Onchorhynchus tshawytscha* may be correlated with significant differences in hearing sensitivities (Osman et al., 2007). Those authors hypothesized that in less dense sagittae, the lag of sagitta movement relative to the motion of the sensory epithelium in response to a certain frequency is attenuated, thereby causing less stimulation than denser sagittae. Accordingly, the increased weight

### Table 7

| Frequency (Hz) | Cave | Tampico | Rio Oxolotán |
|---------------|------|---------|--------------|
|               | SPL threshold (db re 1 μPa) | Mag a threshold (db re 1 μm/s²) | SPL threshold (db re 1 μPa) | Mag a threshold (db re 1 μm/s²) | SPL threshold (db re 1 μPa) | Mag a threshold (db re 1 μm/s²) |
| 100           | 87.2 ± 1.61 | 48.2 ± 1.61 | 88.0 ± 1.48 | 49.0 ± 1.48 | 88.0 ± 1.53 | 49.0 ± 1.53 |
| 200           | 76.2 ± 1.23 | 36.2 ± 1.23 | 78.9 ± 1.87 | 38.9 ± 1.87 | 82.3 ± 4.33 | 42.3 ± 4.33 |
| 300           | 82.1 ± 1.33 | 47.2 ± 1.33 | 82.8 ± 1.12 | 47.6 ± 1.12 | 82.0 ± 2.31 | 47.0 ± 2.31 |
| 500           | 103.6 ± 0.94 | 62.6 ± 0.94 | 103.6 ± 1.53 | 62.6 ± 1.53 | 103.3 ± 2.60 | 62.3 ± 2.60 |
| 1000          | 121.4 ± 0.87 | 88.4 ± 0.87 | 116.6 ± 1.24 | 83.6 ± 1.24 | 116.3 ± 2.03 | 81.3 ± 2.03 |
| 1500          | 128.2 ± 0.90 | 103.2 ± 0.90 | 127.2 ± 1.27 | 102.2 ± 1.27 | 126.0 ± 1.53 | 101.0 ± 1.53 |

**Table 8**

General linear model (GLM) using ‘frequency’ as repeated measurements (Rm). The GLM was calculated with either sound pressure level (SPL) or particle acceleration level (Mag a) as dependent variable. Significant P-values are bold. The same values for mean squares, F and P were obtained regardless of the dependant variable used in the model (‘SPL’ or ‘Mag a’).

| Source | df | Mean square | F | P |
|--------|----|-------------|---|---|
| Between-subjects effects | | | | |
| SL | 1 | 14.52 | 0.43 | 0.52 |
| Population | 2 | 0.39 | 0.01 | 0.99 |
| Sex | 1 | 1.15 | 3.41 | 0.08 |
| Population × sex | 2 | 0.07 | 0.002 | 0.10 |
| Error | 23 | 33 | |
| Within-subjects effects | | | | |
| Rm (frequency) | 1 | 1760.89 | 69.30 | <0.0001 |
| Rm × SL | 1 | 181.83 | 7.16 | 0.014 |
| Rm × population | 2 | 39.70 | 1.56 | 0.23 |
| Rm × sex | 1 | 0.34 | 0.01 | 0.91 |
| Rm × population × sex | 2 | 29.79 | 1.17 | 0.33 |
| Error | 23 | 25 | |
per area of asterisci and lapilli in cave fish may enhance the stimulation of the sensory hair cells.

The differences in otolith morphology between cave and surface fish may reflect different metabolisms. In contrast to surface-dwelling fish from Rio Oxolotán and Tampico, cave fish must spend much of their time and energy to cope with the hydrogen sulfide and the correlated hypoxia by showing aquatic surface respiration (Tobler et al., 2009). Although fish from chamber XIII do not have to cope with this toxin, they show a similarly poor body condition as fish from the other cave chambers. In general, cave fishes exhibit a different (slower) metabolism (cave-dwelling amylloids: Poulsom, 1963; Nemacheilus evezardi: Pati and Agrawal, 2002), and an altered metabolism might affect otolith mineralization. For example, oxygen consumption — used as a proxy for metabolic rate in juvenile Atlantic salmon parr (Salmo salar) and larval zebrafish (Danio rerio) — was positively correlated with sagitta size (Bang and Grønkjær, 2005; Wright, 1991). Otolith mineralization and the role of proteins are still poorly understood (Allemand et al., 2007; Grønkjær, 2005; Wright, 1991). Otolith mineralization and the role of proteins are still poorly understood (Allemand et al., 2007; Grønkjær, 2005; Wright, 1991).

4.3. Acoustic communication

Vocalizations may play an important role in intra-specific communication in fishes (for reviews see Ladich and Myrberg, 2006; Myrberg and Lugli, 2006; Parmentier and Diogo, 2006; Wysocki, 2006). This could be particularly important in a lightless habitat where fish are unable to communicate visually. Interestingly, neither surface nor cave mollies appear to produce species-specific sounds, indicating that sounds might be less important in communicating with conspecifics in this teleost family. One explanation is that the well-developed cephalic lateral line (cf. Parzefall, 1970, 2001) compensates for the lack of visual communication in caves. Although we observed the full range of agonistic and mating behavior in mollies during our laboratory survey, we cannot completely exclude the possibility that surface and/or cave fish might produce sounds in the field (i.e., in another social and/or environmental context), as has been reported in cyprinodontids (Johnson, 2000; Nicoletto and Linscomb, 2008).

5. Conclusions

The observed differences in otolith shape, in the otoliths’ macula-oriented faces and in the weight of asterisci and lapilli in cave fish apparently do not affect hearing sensitivities, at least in terms of auditory thresholds. Cave life and an altered metabolism may be phenotypically plastic.

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