Differences in otolith morphologies between surface- and cave-dwelling populations of *Poecilia mexicana* (Teleostei, Poeciliidae) reflect adaptations to life in an extreme habitat

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**ABSTRACT**

**Questions:** Do otolith morphologies differ between surface- and cave-dwelling forms of *Poecilia mexicana*? Do otolith contours show differences between populations and do they display a morphological gradient from the cave outflow to the innermost cave chamber?

**Organism:** Surface- and cave-dwelling forms of a freshwater fish, the Atlantic molly (*Poecilia mexicana*).

**Field sites:** Two sites in the sulphidic cave system Cueva del Azufre in Tabasco, Southern Mexico, and two sites outside the cave – the cave outflow and a freshwater brook.

**Methods:** We analysed otolith morphology using scanning electron microscopy and otolith contours by Fourier shape analysis (*N* = 67).

**Results:** The otoliths of the cave form of *P. mexicana* are heavier and have a deeper sulcus (= furrow on the inner otolith face) than those of surface dwellers. Otolith contours show a morphological gradient that concurs with the morphocline of other traits (e.g. eye size).

**Keywords:** cave fish, hearing adaptation, morphocline, otolith, sulcus morphology.

**INTRODUCTION**

Cave systems represent extreme habitats due to permanent darkness, and thus lack of visual information (Poulson, 2001). As a result, life in caves requires special adaptations such as the improvement of senses other than the visual system (cf. Poulson, 2001). Studies on cave fish generally focus on the reduction of the visual sense (e.g. Jeffery, 2005; Espinasa and Jeffery, 2006; Strickler et al., 2007) or improvement of the lateral line system (Montgomery et al., 1995, 2001). Apart from the visual sense and the lateral line system, however, fishes are able to perceive their environment through the sense of hearing (e.g. Popper and Lu, 2000). In this study, we assess
whether otolith morphology has changed in cave fish compared with their surface-dwelling ancestors. Otoliths are massive calcium carbonate concretions positioned in sac-like extensions of the membranous part of the inner ear in teleost fishes. In general, otolith morphology is closely connected to physiological functions – that is, the sense of hearing and the vestibular sense (= sense of balance). For example, the depression on the inner (medial) face of the saccular otolith, the sulcus acusticus, is closely connected to the sensory epithelium (Dunkelberger et al., 1980; Popper and Lu, 2000). Changes in hearing ability are reflected in sulcus morphology (e.g. Ramcharitar et al., 2004); however, details of this interrelationship remain poorly understood.

In the fossil record, otoliths are often the sole remains of fishes, and thus represent important data in the reconstruction of ancient aquatic ecosystems. Yet, fossil otoliths have rarely been used in the detection of physiological adaptations such as improved hearing capabilities. This may be due to the scarcity of studies dealing with morphological changes in extant otoliths with regard to inner ear physiology. As a result, our study may also contribute to a better understanding of paleoecology and evolution in teleost fishes.

As a well-investigated model system, we chose the cyprinodontiform Poecilia mexicana (Atlantic molly) from Southern Mexico (Cueva del Azufre) that occurs in the form of a cave-dwelling and several surface-dwelling morphs (e.g. Gordon and Rosen, 1962; Plath et al., 2007a, 2007b, 2007c; see also Tobler et al., 2007). In surface-dwelling P. mexicana, the visual sense plays an important role in communication and predator avoidance (Riesch et al., 2006a, 2006b; Parzefall et al., 2007). The eyes of the cave fish are reduced albeit still functional, while the senses of taste and touch are improved (Gordon and Rosen, 1962; Parzefall, 2001; Körner et al., 2006). The senses of taste and touch, and also the lateral line system, allow for the perception of the environment in the proximity of the fish (e.g. Montgomery et al., 1995, 2001; Popper and Lu, 2000). In contrast, the sense of hearing acts over distances of many metres, and likely provides the fish with an acoustic impression (auditory scene) of the environment, such as a cave system (Popper, 1980; Ladich and Popper, 2001).

The morphcline of Poecilia mexicana in the Cueva del Azufre system

The Cueva del Azufre system is a unique model because of the patchiness of distinctly different habitat conditions within a restricted area (Tobler et al., 2006). Previous morphological analyses have shown that features such as eye reduction reveal a gradient (Gordon and Rosen, 1962; Parzefall, 2001). Compared with the normally developed eyes in the surface fish from the freshwater brooks, fish from the cave outflow have slightly reduced eyes; furthermore, eye reduction gradually progresses in fish from the outermost to the innermost cave chambers. A recent molecular study (Plath et al., 2007b) indicates that the morphological gradient is not due to bidirectional gene flow as previously suggested (Gordon and Rosen, 1962; Parzefall, 2001). Rather, Plath et al. (2007b) found evidence for unidirectional gene flow from the innermost cave chamber in the direction of the outflow.

We assume that this morphological gradient in fish from the Cueva del Azufre system is reflected in the saccular otoliths. The saccular otolith is the largest of the three otolith types in most teleosts (e.g. Assis, 2005), species-specific, and hence may provide insights into phylogenetic relationships between teleost fishes (Nolf, 1985, 1993; Nolf and Tyler, 2006). Moreover, analysis of otolith contours is an important tool for stock discrimination in marine fisheries (e.g. Campana and Casselman, 1993; Stransky et al., 2007, 2008). Otolith contours may also indicate inter-population differences in freshwater fishes (Schulz-Mirbach et al., 2006; Reichenbacher et al., 2007).
Here we present distinct differences in otolith morphology between surface- and cave-dwelling forms of *Poecilia mexicana*. Moreover, we show that results obtained by otolith shape analysis indicate a differentiation between populations and concur with the morphocline of other traits.

**MATERIALS AND METHODS**

**Study system: habitat differences**

We investigated four localities that differ in the degree of exposure to light and hydrogen sulphide concentration (for details, see Gordon and Rosen, 1962; Tobler et al., 2006):

- cave chamber X of the Cueva del Azufre (the innermost chamber in this study);
- cave chamber V (the outermost chamber in this study);
- the cave outflow (El Azufre); and
- an adjacent freshwater brook (Arroyo Cristal)

Although Arroyo Cristal and El Azufre flow into the same river (Río Oxolotan), they are separated by a waterfall (Fig. 1) (Plath et al., 2007b). In general, the cave water shows varying hydrogen sulphide concentrations and is essentially dark, while the cave outflow is characterized by lower concentrations of this toxin and is under the influence of light. In contrast to cave chamber X, cave chamber V is not completely dark because light penetrates through several openings in the ceiling of the front cave chambers. In addition, the concentration of hydrogen sulphide in cave chamber V is comparable to that in El Azufre (cave outflow), whereas cave chamber X is characterized by high concentrations of this toxin. Arroyo Cristal is a pure freshwater brook without hydrogen sulphide and is exposed to normal solar radiation.

In contrast to other cave systems that are characterized by a scarcity of food due to the lack of photosynthesis (see Poulson, 2001), the food web in the Cueva del Azufre system is based on chemoautotrophic bacteria using the hydrogen sulphide (see Tobler et al., 2006). In general, hydrogen sulphide is toxic for metazoans such as vertebrates by hampering pulmonary functions and inhibiting proteins involved in the respiratory chain (Bagarinoa, 1992; Grieshaber and Völkel, 1998). The forms of *Poecilia mexicana* that live in the Cueva del Azufre and the cave outflow are able to cope with the hydrogen sulphide. However, it would appear that the toxin has extensive effects on the physiology and the behaviour of these fish (see Plath et al., 2007a). The fish display a considerably lower body condition compared with populations from the freshwater brooks (Tobler et al., 2006). Moreover, fish living in hydrogen sulphide-enriched water display a special behaviour for detoxification, aquatic surface respiration, which appears to be energetically costly (Plath et al., 2007a).

**Sample collection and otolith preparation**

Altogether, 67 specimens of *Poecilia mexicana* Steindachner, 1863 were collected at the above-mentioned sites (Table 1, Fig. 1). Fish were stored in 99.8% ethanol until dissection of the otoliths. The standard length (SL) of the fish was measured to the nearest millimetre. Exclusively adult specimens were used with standard length > 30 mm and otolith length ($L_\text{ot}$) > 1000 µm. To minimize size-dependent shape variation in the analyses of the
otolith contour, exceptionally large specimens were also excluded. A range of otolith lengths from 1000 to 1700 µm with corresponding standard lengths of about 30–50 mm was used.

Skulls were opened ventrally and left and right otoliths were dissected. The otoliths were cleaned in a 1% potassium hydroxide solution for 4 h, soaked with distilled water for 12 h,
Otolith morphology in cave mollies

Table 1. Synopsis of data about the localities in and around the Cueva del Azufre system

<table>
<thead>
<tr>
<th>Localities</th>
<th>No.</th>
<th>L_{ot} range [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arroyo Cristal (freshwater brook)</td>
<td>9</td>
<td>1023–1510</td>
</tr>
<tr>
<td>El Azufre (cave outflow)</td>
<td>22</td>
<td>1231–1546</td>
</tr>
<tr>
<td>Cave chamber V (Cueva del Azufre)</td>
<td>22</td>
<td>1058–1586</td>
</tr>
<tr>
<td>Cave chamber X (Cueva del Azufre)</td>
<td>14</td>
<td>1076–1599</td>
</tr>
</tbody>
</table>

Note: No., number of left otoliths; L_{ot}, length of left otoliths.

and stored dry. Otoliths were weighed (W_{ot}) with a Sartorius 2405 scaled (± 0.001 mg, S.D.) to the nearest 0.01 mg. Maximum length (L_{ot}) and height (H_{ot}) were measured under a stereomicroscope using the Leica® Image Software (IMAGIC 1000), and a digital sliding calliper (± 2 µm, S.D.). Qualitative and quantitative analyses were based on exclusively left otoliths.

Otolith morphology

Note that we distinguish between ‘otolith contour’ and ‘otolith morphology’, as the latter comprises the overall contour as well as the sulcus morphology (see Gauldie and Crampton, 2002). Qualitative characters from all otoliths were analysed with a stereomicroscope. Representative otoliths from each locality were investigated using a scanning electron microscope, a LEO 1430 VP at the Zoological State Collection (Munich, Germany). Electroscope images were taken of the inner face of the otoliths to study the otolith contour and, in particular, details of the sulcus morphology.

Regarding the inner face of the otoliths, we focused on two features: (i) deepness of the sulcus and (ii) presence or absence of a small impression in the upper posterior part (= cauda; see Fig. 2) of the sulcus (Fig. 3A<sub>2</sub>, B<sub>2</sub>: impression present; Fig. 3C<sub>2</sub>, D<sub>2</sub>: impression absent). We chose the feature ‘impression’ because its absence was mostly associated with the development of a deep sulcus. We compared the relative frequencies of the presence of the impression between sites using a χ<sup>2</sup>-test. The deepness of the sulcus was determined qualitatively. We classified a sulcus as flat if it corresponded to that seen in Fig. 3A<sub>2</sub>, and deep if it resembled that seen in Fig. 3C<sub>2</sub> or D<sub>2</sub>.

Otolith ratios

The height to length (H_{ot}/L_{ot}) ratio represents a simple shape descriptor that is widely used in studies of extant and fossil otoliths (e.g. Weiler, 1963; Reichenbacher and Sienknecht, 2001; Stransky and MacLellan, 2005). We therefore included this ratio in our study of the otolith contour. The ratio data did not deviate from the assumptions of normal distribution (Shapiro Wilk test, P > 0.5) and homogeneity of variances (Levene test, P > 0.5). A one-way analysis of variance and an all-pairwise post hoc test (Bonferroni, α-level = 0.05) were conducted for comparison of means between the localities.

We calculated the weight to length (lnW_{ot}/lnL_{ot}) ratio as a descriptor of the third dimension of otoliths. We also included the otolith weight to infer changes in otolith morphology as a result of possible adaptation to extreme habitat conditions. The ratio
showed significant deviations from the assumptions of normal distribution (Shapiro Wilk test, \( P < 0.05 \)) and homogeneity of variances (Levene test, \( P < 0.05 \)). Therefore, we employed a non-parametric Mann-Whitney \( U \)-test to compare ratios among sites and corrected \( \alpha \)-levels (0.05) for multiple comparisons with Bonferroni adjustments.

**Otolith contour (Fourier shape analysis)**

Otoliths were positioned with their outer face on plasticine, such that they were lying plainly under the dissecting scope with the sulcus oriented horizontally. Digital images were taken at 76 to 122× with a CCD-camera connected to a PC using the Leica® Image Software (IMAGIC 1000). Otolith images were focused on the outermost contour. All digital images were processed in Adobe Photoshop CS2 by generating a contrast of 100% along the contour (white object on black background). Contours of otoliths were digitized in tpsDig2 (Rohlf, 2004) and raw x-y coordinates were saved.

For Fourier shape analysis of the digitized otolith contours, the HSHAPE software (Crampton and Haines, 1996) consisting of three programs (HANGLE, HMATCH, and HCURVE) was used. In this type of Fourier transformation, Fourier functions are fitted to a function of the tangent angle dependent on arc length (Haines and Crampton, 2000), and calculated with
the Fast Fourier Transform (FFT) algorithm. Normalization of size was performed automatically in HANGLE (harmonics: 20; smoothing iterations: 10), and normalization of orientation was done in HMATCH for the entire sample set. The amplitude versus harmonic number plot indicated that at least 20 harmonics were necessary for the analysis. Consequently, the Fourier analysis yielded 38 Fourier descriptors for each otolith contour.

Fig. 3. Scanning electron microscope images of left otoliths of (A₁–A₃) Arroyo Cristal, (B₁–B₃) El Azufre, (C₁–C₃) cave chamber V, and (D₁–D₃) cave chamber X. Images A₂–D₂ focus on the sulcus, while A₁–D₁ show details of the centre of the sulcus. Black arrows (C₁, D₁) indicate the bulging crista superior and the hollow-like area above this crista. White arrows (A₂, B₂) mark the small impression in the upper posterior part of the sulcus. Thin white arrows (A₃) indicate the concentric symmetry of crystallites. Scale bars equal 500 µm (A₁–D₁), 100 µm (A₂–D₂), and 20 µm (A₃–D₃), respectively.
Average contours were calculated to visualize differences of the otolith contours among populations. The Fourier descriptors for each population were averaged and then 1025 x-y values were back-calculated in HCURVE and plotted.

Statistical analyses

The Fourier descriptors were subjected to a principal components analysis (PCA). A variance–covariance-based PCA was calculated in NTSYS-pc 2.2 (Rohlf, 2005). The number of principal components necessary to explain more variance than expected by chance was determined using the ‘broken stick model’ according to Jackson (1993, p. 2207). Synthetic model shapes for those principal components relevant under the ‘broken stick model’ were calculated following the procedure described in Haines and Crampton (2000). The 95% confidence ellipses of the means and groups were calculated according to the equations given in Sokal and Rohlf (1995, pp. 589–591).

Subsequently, a canonical discriminant analysis (CDA) was conducted based on the extracted and interpreted principal components as variables to assess differences between populations. The CDA was processed in SPSS 15.0 (SPSS, Inc., 2006) with an equal a priori probability for all groups. The quality of classification was tested by jackknifed cross-validation. We plotted the 95% confidence ellipses for the discriminant plot in SYSTAT 9 (SPSS, Inc., 1999), and calculated the contribution of the contour features to each discriminant function and to the overall discrimination according to the equation given in Backhaus et al. (2006, p. 188).

RESULTS

Sulcus morphology

The otoliths of *Poecilia mexicana* from the four sites clearly differed with regard to sulcus morphology (for a definition of terms, see Fig. 2). In general, the sulcus of the surface populations El Azufre and Arroyo Cristal was flat, characterized by the presence of a small impression located in the upper part of the cauda (Fig. 3A₂, B₂), and often showed a concentric symmetry of crystallites in its central part (Fig. 3A₃, B₃ vs. C₃, D₃). This complement of sulcus traits is similar to that seen in otoliths of the closely related species *P. formosa* and *P. latipinna* (results not shown). Conversely, only 14% of the otoliths from cave chamber V and 18% of the otoliths from cave chamber X displayed a small caudal impression (Table 2). In addition, 86% of the otoliths from cave chamber V and 79% from cave chamber X possessed a deepened sulcus (Table 2). The deepening mainly appeared at the posterior tip of the cauda and the anterior tip of the ostium. The four populations differed significantly from one another in the absence/presence of this small caudal impression (χ² = 25.95; d.f. = 3; P < 0.001), and this difference remained significant if the Arroyo Cristal population was excluded (χ² = 13.39; d.f. = 2; P < 0.01). In general, the development of a deep sulcus and absence of the small impression were most often accompanied by a thick and bulging crista superior and a deepened area above the crista (Fig. 3C₁, D₁).
The height to length ratio (Fig. 4A) differed significantly among populations \((F_{3,63} = 6.61; P = 0.001)\). A post hoc pairwise comparison revealed that the Arroyo Cristal population differed from all other sites \((P < 0.05)\). No significant differences were observed when only the sample sites in the cave system were compared (El Azufre and both cave chambers; \(F_{2,55} = 1.86; P = 0.166\)). Otoliths from cave chamber X, however, tended to be more elongated than those from the cave outflow and cave chamber V. In general, the height to length ratio revealed a shift from rather short otoliths in Arroyo Cristal to the elongated otoliths found in cave chamber X.

The weight to length ratio (Fig. 4B) revealed a significant difference between the cave outflow (El Azufre) and the innermost cave chamber X \((P = 0.012)\). The other comparisons between populations were not significant \((P > 0.05)\). In general, Arroyo Cristal, El Azufre, and cave chamber V showed similar weight to length ratios, whereas cave chamber X displayed a trend towards heavier otoliths.

Qualitative characterization of the otolith contours (Figs. 2, 3)

In the following, five characters of the otolith contour are briefly described that differed between populations.

- **Arroyo Cristal** (Fig. 3A): (1) overall contour: trapezoid; (2) excisura: moderately incised; (3) rostrum: distinct; pointed or rounded rectangular; (4) antirostrum: distinct; rounded, rarely pointed; (5) posterodorsal edge: well developed; tip-like.

- **El Azufre** (Fig. 3B): (1) overall contour: trapezoid, rarely rounded triangular; (2) excisura: slightly to moderately incised; (3) rostrum: distinct; pointed or rounded rectangular; (4) antirostrum: distinct, rarely weakly developed; rounded or pointed; (5) posterodorsal edge: slightly to well developed; rounded or tip-like.

- **Cave chamber V** (Fig. 3C): (1) overall contour: trapezoid or rounded triangular; (2) excisura: slightly to distinctly incised; (3) rostrum: distinct; tip-like or rectangular; slightly dorsally bent; (4) antirostrum: weakly or distinctly developed; often tip-like and dorsally bent; (5) posterodorsal edge: weakly to moderately developed, rarely distinct; rounded or tip-like.
Cave chamber X (Fig. 3D): (1) overall contour: trapezoid or triangular; (2) excisura: slightly to distinctly incised; (3) rostrum: distinct; tip-like or rectangular; often slightly dorsally bent; (4) antirostrum: weakly or distinctly developed; often tip-like and dorsally bent; (5) posterodorsal edge: moderately to well developed; rounded or tip-like.

Principal components analysis (PCA) and interpretation of the morphospace

The first six principal components (PCs) were relevant under the ‘broken stick model’ and explained 61.2% of the overall variance. The first three principal components accounted for 43.1%, whereas PC4–PC6 only explained an additional 18.1% of the variance (Table 3). The three-dimensional plot of the first three principal components revealed a slight differentiation of populations despite a relatively large overlap between populations (Fig. 5). This differentiation was even more obvious if only the PC 1 vs. PC2 (Fig. 6A) or the
<table>
<thead>
<tr>
<th>Principal component (explained variance in %)</th>
<th>1 (21.8)</th>
<th>2 (11.8)</th>
<th>3 (9.5)</th>
<th>4 (6.8)</th>
<th>5 (6.1)</th>
<th>6 (5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explained morphological character</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arroyo Cristal</td>
<td>slight to moderate</td>
<td>distinct</td>
<td>moderately to distinctly tip-like</td>
<td>distinctly posterior</td>
<td>tip-like to bilobate</td>
<td>tip-like</td>
</tr>
<tr>
<td>El Azufre</td>
<td>slight to (moderate)</td>
<td>slight to distinct</td>
<td>slightly bilobate to distinctly tip-like</td>
<td>distinctly posterior</td>
<td>intermediate</td>
<td>round to tip-like</td>
</tr>
<tr>
<td>Cave chamber V</td>
<td>slight to distinct</td>
<td>moderate</td>
<td>moderately to distinctly bilobate</td>
<td>moderately to distinctly posterior</td>
<td>intermediate</td>
<td>round to tip-like</td>
</tr>
<tr>
<td>Cave chamber X</td>
<td>slight to moderate</td>
<td>moderate to distinct</td>
<td>slightly tip-like to moderately bilobate</td>
<td>moderately posterior</td>
<td>moderately to distinctly bilobate</td>
<td>trend to the tip-like form</td>
</tr>
</tbody>
</table>

*PC1 additionally explains the degree of the bilobate shape of the posteroventral edge.* *PC3 explains to a limited extent the development of the antrostrum.*
PC 2 vs. PC 3 plots (Fig. 6B) were considered. In the PC 1 vs. PC 2 plot, the 95% confidence ellipses of the means allowed a distinction between (1) Arroyo Cristal and El Azufre and (2) cave chambers V and X with respect to the PC 2 axis (Fig. 6A). In the PC 2 vs. PC 3 plot (Fig. 6B), all four populations were separated. In this plot, Arroyo Cristal and cave chamber X were almost completely separated with respect to their 95% confidence ellipses of the groups.

A detailed interpretation of the principal components is given in Table 3 and Fig. 7. Four of the six principal components (PC2, PC 4–6) explained features of the posterodorsal edge and the dorsal rim; the remaining two principal components accounted for the deepness of the excisura (PC1) and shape of the posteroventral edge (PC3). The characterization of the otoliths from the four populations was in concordance with the qualitative description of the traits ‘excisura’ and ‘posterodorsal edge’ (see above). The distinctness of the posteroventral edge had not previously been identified in the qualitative comparison as a morphologically important feature of the contour.

**Canonical discriminant analysis (CDA) and population differentiation**

The discriminant plot (Fig. 8) and jackknifed classification matrix (Table 4) confirmed the degree of separation indicated by the PCA. Arroyo Cristal was distinctly different from
the cave chambers. Otoliths from Arroyo Cristal and the cave outflow (El Azufre) showed some overlap in the discriminant plot and some misclassifications between the latter two populations were detected. In the discriminant plot (Fig. 8), El Azufre was positioned intermediately between the freshwater brook Arroyo Cristal and cave chambers, in particular with respect to discriminant axis 1. The cave chambers showed pronounced, large overlap and a high rate of misclassification. Otoliths of the cave chambers were misclassified to a lesser extent into the cave outflow El Azufre.

Fig. 6. Plots of the 95% confidence ellipses of the means (shaded) and the groups (dashed lines) for (A) PC1 vs. PC2 and (B) PC2 vs. PC3.
Fig. 7. Synthetic model shapes calculated for the first six principal components (PC) according to Haines and Crampton (2000). PC2–PC5 are each plotted vs. PC1. The open arrows indicate the morphological feature explained by PC1. Black arrows indicate the morphological features explained by PC2–PC6 (see also Table 3).
The pattern of similarity/dissimilarity between the four localities can be explained by the contribution of the previously interpreted principal components to each discriminant axis and to the overall discrimination (Table 5). The development of the posterodorsal edge and the shape of the posteroventral edge discriminated most strongly among the populations along discriminant function 1, in particular between Arroyo Cristal and the cave chambers. The excisura largely contributed to discriminant function 2, thereby moderately separating Arroyo Cristal vs. El Azufre, and cave chambers V vs. X. In general, these characters (included in the first three principal components) contributed most strongly to the

**Fig. 8.** Discriminant function scores for the first six principal components relevant under the ‘broken stick model’ (Jackson, 1993). Ellipses represent 95% confidence ellipses of the groups.

**Table 4.** Jackknifed classification matrix of the canonical discriminant analysis between the four localities

<table>
<thead>
<tr>
<th>Locality</th>
<th>Arroyo Cristal</th>
<th>El Azufre</th>
<th>Cave chamber V</th>
<th>Cave chamber X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arroyo Cristal</td>
<td>77.8 (7)</td>
<td>22.2 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>El Azufre</td>
<td>9.1 (2)</td>
<td>77.3 (17)</td>
<td>0 (0)</td>
<td>13.6 (3)</td>
</tr>
<tr>
<td>Cave chamber V</td>
<td>0 (0)</td>
<td>13.6 (3)</td>
<td>63.6 (14)</td>
<td>22.7 (5)</td>
</tr>
<tr>
<td>Cave chamber X</td>
<td>0 (0)</td>
<td>7.1 (1)</td>
<td>28.6 (4)</td>
<td><strong>64.3</strong> (9)</td>
</tr>
</tbody>
</table>

*Note:* The percentages in rows represent the classification into the localities given in columns; the corresponding number of specimens is given in parentheses. The percentages of correctly classified individuals are shown in *bold*. Overall classification success is 70.1% (Wilks’ $\lambda = 0.137$).

The pattern of similarity/dissimilarity between the four localities can be explained by the contribution of the previously interpreted principal components to each discriminant axis and to the overall discrimination (Table 5). The development of the posterodorsal edge and the shape of the posteroventral edge discriminated most strongly among the populations along discriminant function 1, in particular between Arroyo Cristal and the cave chambers. The excisura largely contributed to discriminant function 2, thereby moderately separating Arroyo Cristal vs. El Azufre, and cave chambers V vs. X. In general, these characters (included in the first three principal components) contributed most strongly to the
discrimination among populations. The development of the posterodorsal edge (PC2) represented the most important discriminatory factor (Table 5).

**DISCUSSION**

We analysed sulcus morphology, otolith weight, and otolith contour in surface- and cave-dwelling *Poecilia mexicana* to determine whether (i) extreme habitat conditions affect otolith morphology and (ii) otolith contours allow differentiation between populations in the sense of a morphocline. Our results indicate that:

1. *Poecilia mexicana* from the sites exposed to light (Arroyo Cristal, El Azufre) reveal otoliths with a flat sulcus and a small impression is present, while the cave fish possess otoliths characterized by a trend towards formation of a deep sulcus and the lack of a small impression.
2. Otolith weight in fish from cave chamber X (innermost chamber) is significantly higher than that in fish from El Azufre (cave outflow).
3. Otolith contours (i) are distinctly different between the fish from Arroyo Cristal (freshwater brook) and the cave chambers; (ii) indicate that the El Azufre (cave outflow)
population is intermediate between those of Arroyo Cristal and the cave, but more similar to otoliths from the cave fish; and (iii) reveal only slight differences among the fish from the cave chambers.

**Do changes in otolith morphology reflect an adaptation to darkness?**

A recent study focusing on silver perch (*Bairdiella chrysoura*) revealed that a close relationship exists between specialization in the anatomy of the inner ear, otolith morphology, and a well-developed hearing ability (Ramcharitar et al., 2004). One of the most intriguing facts concerning the saccular otoliths of the silver perch is the deeply grooved sulcus and increased overall thickness of the otoliths (Ramcharitar et al., 2004, Figure 3A). These specializations resemble those observed in the otoliths of the cave molly. Ramcharitar et al. (2004) suggested that the deepness of the sulcus may lead to a different orientation pattern of the hair cells of the sensory epithelium and a different stiffness of the otolithic membrane that could cause differences in ability to localize sound sources. This would fit well with the suggestion of improved hearing in cave mollies.

It is assumed that otolith weight can indicate changes in hearing capability as well (see Ramcharitar et al., 2001, 2004). In general, three main factors may influence the weight of cave molly otoliths: (i) extrinsic factors (hydrogen sulphide, temperature, pH); (ii) body condition of the fish connected to an indirect influence of the toxin hydrogen sulphide; and (iii) adaptation to darkness.
(i) Hydrogen sulphide may alter biomineralization processes in the otoliths because it affects important proteins involved in this process (see Bagarinao 1992). This may have resulted in different otolith weights among fish living in pure freshwater (Arroyo Cristal) and those living under the influence of this toxin. Little is known about proteins involved in the biomineralization of otoliths (for a recent overview, see Söllner and Nicolson, 2004). Therefore, further interpretations of our data with regard to hydrogen sulphide are not possible at present.

Apart from hydrogen sulphide, temperature and pH may also influence otolith weight. An increase in temperature may boost the biomineralization processes in otoliths, and thus boost otolith growth (Lombarte and Lleonart, 1993). However, temperature in the cave habitats and the outflow is constant throughout the year (28°C), while the pH shows only slight differences (6.7–7.1) (Tobler et al., 2006). It is therefore unlikely that temperature or pH can explain the differences in otolith weight.

(ii) Fish exposed to hydrogen sulphide show a lower body condition than those from Arroyo Cristal (Tobler et al., 2006). The cave fish have to invest large amounts of energy to detoxification, which may result in their low body condition despite the abundance of readily available food (Tobler et al., 2006; Plath et al., 2007a). In a non-sulphidic cave system situated near to the Cueva del Azufre, a similar low body condition occurs in Poecilia mexicana because of restricted food supply (starvation) (Tobler et al., 2007). Starvation leads to narrowing of microincrements – that is, less material is incorporated into the otolith [Massou et al. (2002), experiments with Nile tilapias]. Therefore, if there were a considerable influence of low body condition, otoliths of cave fish that have to cope with the highest hydrogen sulphide concentrations (chamber X) should possess the least heavy otoliths. However, the opposite was observed: otoliths from the innermost cave chamber were the heaviest.

(iii) Principally, maximum growth of the otolith occurs on the inner face (the ‘sulcus face’) that is connected to the sensory epithelium (Allemand et al., 2007). Accordingly, the deepened sulcus, as observed in P. mexicana cave forms, may result from an increased accumulation of material around the sulcus. It is therefore conceivable that the depth of the sulcus and the otolith weight are correlated to some extent. A ‘greater otolithic mass is predicted to confer higher sensitivity to low-frequency sounds’ (Ramcharitar et al., 2004). We therefore suggest that the increased otolith weight, together with the deepened sulcus, may point towards an adaptation to life in darkness with improved hearing abilities.

It is well known that the visual component plays an important role in the social behaviour of P. mexicana populations inhabiting surface habitats (Rausch et al., 2006b). The improved senses of taste and touch, together with a more effective lateral line system, are suitable for interactions with conspecifics over short distances (Plath et al., 2004, 2006). To detect more distant sound sources, the sense of hearing plays an important role (Popper and Lu, 2000). Therefore, we assume that a cave fish would benefit from improved hearing capabilities that allow for a better orientation in the dark environment. Studies on deep-sea fish species have demonstrated that the absence of light leads to specializations of the inner ear (e.g. Popper, 1980; Buran et al., 2005; see also Lombarte and Cruz, 2007). These specializations may effectively enhance hearing capabilities, and thus compensate for the lack of visual information.

**Population differentiation and the morphocline**

Morphological traits (e.g. eye size) were found to display a morphological gradient (‘morphocline’) from the outflow of the cave (El Azufre) to the ‘true’ innermost cave chamber XIII.
Stabilization of this morpholine was explained by bidirectional gene flow between the cave outflow and cave chambers (Gordon and Rosen, 1962; Parzefall, 2001). An investigation of population differentiation using microsatellite markers, however, revealed that gene flow between the outflow and the cave is unidirectional – that is, from the cave towards the outflow. Bidirectional gene flow occurs only between the different cave chambers (Plath et al., 2007b). Otoliths express the morphological gradient from the outflow to the innermost cave chamber (chamber X) in a trend with the height to length ratio. The multivariate analyses of the otolith contours, indicating large overlap between the two cave chambers, provide support for the assumed extensive bidirectional exchange between the cave chambers (Plath et al., 2007b). The moderate misclassification between otoliths of the outflow and the cave chambers and their proximate position in the PCA reflect a close relationship, which was also detected by the molecular analysis (Fig. 3) (Plath et al., 2007b). In addition, the results indicate that less exchange exists between the cave and the cave outflow because if there were extensive exchange between the outflow and the “outermost” chamber V, one would expect a high misclassification rate similar to that determined between the cave chambers.

The highest hydrogen sulphide concentrations are present in chamber X, while chamber V and the outflow have low to moderate concentrations (for detailed measurements, see Tobler et al., 2006). Perhaps the difference between chamber X and chamber V/outflow in the height to length ratio suggests some kind of indirect effect of this toxin. However, testing of this hypothesis requires further analyses, since the influence of genetic and ecological parameters on otolith contours have yet to be detailed (see Lombarte and Lleonart, 1993; Stransky et al., 2008). Finally, the physiological aspect of otoliths should also be considered when studying the reasons for the different otolith contours among Poecilia mexicana populations. Modifications in otolith contour would lead to changes in the centre of mass, resulting in a different sensitivity of the vestibular sense (Popper et al., 2005).

**SUMMARY**

We found changes in otolith morphology, including sulcus morphology, otolith contour, and otolith weight, between Poecilia mexicana populations from four localities in and around the Cueva del Azufre cave system. Differences in sulcus morphology were most pronounced between surface- and cave-dwelling fish, and a morphological gradient in otolith contour from outside the cave towards the innermost cave chamber was detected. We conclude that the differences in sulcus morphology may indicate an improvement of sound perception in cave-inhabiting populations. We further assume that greater otolith weight in cave mollies (particularly from cave chamber X) is an effect of the increased depth of the sulcus. Increased otolith weight may further improve the sense of hearing. However, the improvement of this sense might still be in progress, because some individuals from the cave exhibit otoliths with a flat sulcus as seen in surface-dwelling fish. Future studies focusing on features of the sensory epithelium and measurements of the hearing capabilities are warranted. The Cueva del Azufre system with its different habitat conditions may provide an excellent case scenario to gain deeper insights into the interrelationships between otolith morphology and aspects of hearing physiology, which continue to be poorly understood. The results of our study and future analyses will also allow for a better understanding of variable sulcus morphologies within a single fossil species (see Reichenbacher and Weidmann, 1992).
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