

A morphological gradient revisited: cave mollies vary not only in eye size

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Abstract In the Mexican Cueva del Azufre, cave-dwelling Atlantic mollies (*Poecilia mexicana*, Poeciliidae) have been reported to exhibit a pronounced gradient in eye size from front cave chambers towards rear cave chambers. Here, we re-examined this morphological gradient using a geometric morphometric analysis of body shape to test for variation along the gradient in traits other than eye size. Our results indicate that fish within the cave also vary in head size. Whereas differences in eye size appear to be related to differences in the amount of light received among cave chambers, variance in head size coincides with differences in hydrogen sulfide concentrations. Consequently, the morphocline within the Cueva del Azufre is more complex than previously assumed and raises interesting new questions on the mechanisms maintaining these morphological variations on such a small spatial scale.

Keywords Cave biology · Environmental gradient · Hydrogen sulfide · Local adaptation · Morphocline · Poeciliidae

Introduction

Poecilia mexicana, the Atlantic molly, is a common freshwater fish along the Atlantic versant of Central America ranging from northern Mexico to Costa Rica (Miller et al. 2005). It is a small-bodied, livebearing species of the family Poeciliidae that commonly inhabits coastal lagoons, lowland ponds, and rivers as well as upland streams. In southern Mexico, *P. mexicana* has also colonized caves and springs rich in hydrogen sulfide (Gordon and Rosen 1962; Tobler et al. 2006, 2008b). These provide environments fundamentally different from other aquatic habitats: (1) the perpetual darkness requires organisms to rely exclusively on non-visual senses for orientation and foraging as well as inter- and intraspecific interactions (Langecker 2000); (2) the presence of hydrogen sulfide (H₂S), which is a potent respiratory toxicant lethal to most metazoans in micromolar amounts (Bagarinao 1992; Grieshaber and Völkel 1998), requires organisms to have strategies in place to either avoid the uptake of the chemical or to detoxify it (Bagarinao 1992; McMullin et al. 2000).

Poecilia mexicana living in cave and sulfidic habitats are locally adapted and diverged phenotypically and genetically from conspecifics inhabiting “normal” freshwater habitats (Plath et al. 2007a; Tobler et al. 2008a). Cave populations are characterized by having reduced eye size and pigmentation, more slender bodies, as well as more elaborate

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chemo- and mechanosensory structures (Fig. 1a; Gordon and Rosen 1962; Walters and Walters 1965; Peters et al. 1973; Parzefall 2001; Tobler et al. 2008a, b). Unlike surface populations, they are able to communicate with conspecifics through non-visual cues (Plath et al. 2004, 2005; Tobler et al. 2008c). Cavefish are also more successful at avoiding predator attacks in darkness (Tobler 2009). Similarly, fish from sulfidic habitats rely on behavioral responses (aquatic surface respiration, during which fish skim the surface of the water where diffusion maintains a better oxygenated layer) to cope with the toxicity of the environment (Plath et al. 2007b). They have evolved enlarged heads and gills that facilitate efficient oxygen acquisition (Tobler et al. 2008a) and possess mechanisms to detoxify sulfide physiologically (Peters et al. 1973; re-analyzed in Plath and Tobler in press).

Phenotypic variation does not only exist among populations living under divergent environmental conditions, but variation in various traits has been documented within the population of the Cueva del Azufre (a sulfidic cave). Already in the first account of the cave and its inhabitants, Gordon and Rosen (1962) mentioned a morphological cline in *P. mexicana*. They reported a steady decrease in eye size, caudal peduncle depth, and the number of scales along a gradient spanning from the front of the cave to the rear-most cave chamber. Other studies have since been repeatedly documenting a morphocline in eye size (e.g., Walters and Walters 1965; Parzefall 1970; Peters et al. 1973; Plath et al. 2007a). Originally, it was hypothesized that the morphocline is generated and maintained by gene flow from adjacent surface habitats towards the inner cave chambers and outwards-directed gene flow from the innermost cave chambers (Gordon and Rosen 1962; Parzefall 1970; Peters et al. 1973; Parzefall 2001). Gordon and Rosen even suspected the existence of a blind source population of *P. mexicana* in unexplored and inaccessible deeper parts of the cave. Evidence from a population genetic study, however, falsified the hybrid origin of the morphocline, as there was no gene flow from surface to cave habitats (Plath et al. 2007a). In this study, the morphocline within the Cueva del Azufre was reexamined using a geometric morphometric approach. Rather than just focusing on eye size, we were testing whether other traits varying among *P. mexicana* populations (e.g., head size or

body depth) also vary within the Cueva del Azufre population.

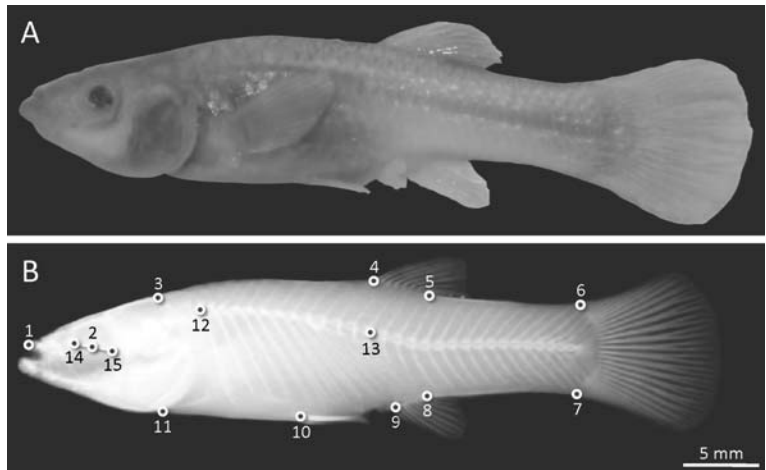
Materials and methods

The Cueva del Azufre is a sulfidic cave located near the village of Tapijulapa in the southern Mexican state of Tabasco (N 17.442 °, W 92.775 °). The cave is only about 500 m deep and structured into different chambers, the nomenclature of which follows Gordon and Rosen (1962; also see Hose and Pisarowicz 1999 for a more detailed map of the cave). The front chambers obtain some dim light through numerous skylights, whereas the rearmost cave chambers are completely dark (Parzefall 2001). The cave is drained by a creek fed by a number of springs throughout the cave, most of which contain high levels of dissolved H₂S (Tobler et al. 2006).

Poecilia mexicana occur throughout the cave, and for this study, they were collected in chambers II, V, X, and XIII. These four chambers differ in environmental conditions (Fig. 2; Tobler et al. 2006, 2008a). The two front-most chambers (II and V) exhibit moderate H₂S concentrations and receive dim light through skylights. Chamber X harbors some of the largest sulfide springs within the cave and has accordingly high H₂S concentrations. Skylights are absent. Finally, chamber XIII is the rearmost cave chamber in the Cueva del Azufre. It is located upstream of the uppermost sulfide springs and thus represents the only aquatic microhabitat within the cave that does not contain sulfide. Like chamber X, this chamber is entirely dark. Furthermore, this chamber is separated from all other chambers by a small waterfall (~1.6 m height).

For the analysis of body shape, lateral radiographs were taken with a Hewlett-Packard (Palo Alto, CA) Faxitron cabinet X-ray system. 13 landmark points were digitized on each image using the software program tpsDig (Rohlf 2004a). Landmarks (see Fig. 1b) included the tip of the upper jaw (1); the center of the orbital (2); the posterodorsal tip of the skull (3); the anterior (4) and posterior (5) junction of the dorsal fin with the dorsal midline; the junction of the caudal fin with the dorsal (6) and ventral (7) midline; the anterior (8) and posterior (9) junction of the anal fin with the ventral midline; the anterior junction of the pelvic fins and the ventral midline

Fig. 1 **a** Female cave molly from cave chamber X of the Cueva del Azufre. Note the small eye and the low level of body pigmentation. **b** Radiograph of a female cave molly (chamber X) with the thirteen landmarks (1–13) and two reference points (14 and 15) used to describe body shape. Detailed information on the location of landmarks and reference points are given in the text



(10); the bottom of the head where the operculum breaks away from the body outline (11); the center of the first vertebra (12); and the center of the third vertebra with a hemal arch (13). Furthermore, eye diameter was measured to the nearest 0.01 mm with calipers. This distance was halved, and used to position two reference points anterior (14) and posterior (15) to the orbit landmark (with the same y-value).

A geometric morphometric analysis was performed based on the coordinates of the digitized landmarks and reference points (e.g., Zelditch et al. 2004). Data were translated to NTS format using tpsUtil (Rohlf 2006). Landmark coordinates were aligned using least-squares superimposition as implemented in the

program tpsRelw (Rohlf 2007) to remove effects of translation, rotation, and scale. The weight matrix of the aligned coordinates plus reference points was calculated with tpsRelw and then subjected to a principal component analysis to reduce the data to true dimensionality. Null dimensions were dropped from the analysis and the remaining principal axes were retained as shape variables. Body shape variation (24 principal components) was analyzed using a multivariate analysis of covariance (MANCOVA). Assumptions of multivariate normal error and homogeneity of variances and covariances were met. *F*-ratios were approximated using Wilks' lambda, effect strengths using partial eta squared (η_p^2). We tested for effects of "centroid size" to control for multivariate

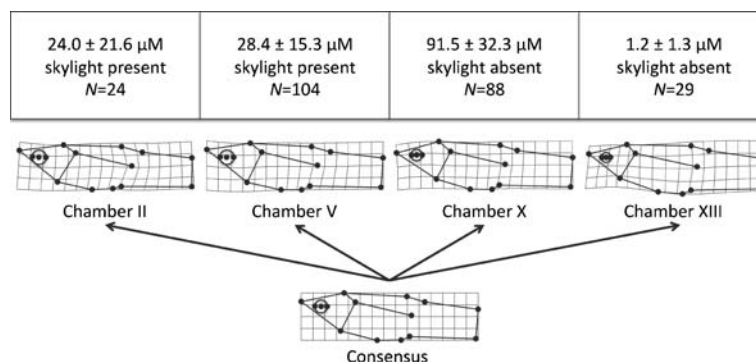


Fig. 2 Cartesian transformation grids that depict the deviation of the population mean (based on estimated marginal means, which controlled for the effects of size and sex) to the overall mean body shape (consensus). Effects are magnified two-fold. In the panel above, basic information on the environmental conditions (H₂S concentrations and presence and absence of

skylights) are reported for the different cave chambers as well as the number of individuals examined from each chamber. H₂S concentrations represent means (± standard deviations) from measurements over multiple years and are taken from Tobler et al. (2008a)

allometry and included “sex” as well as “cave chamber” as independent variables. Shape variation among fish from different chambers was visualized using thin-plate spline transformation grids based on the deviation of estimated marginal means to the consensus configuration using tpsSpln (Rohlf 2004b). To provide a quantitative basis for the nature of shape effects, correlations of superimposed landmark coordinates with the shape gradients were calculated. This was done by creating a score for each specimen on the focal shape axis. To wit, we multiplied the eigenvector of the effect SSCP matrix by the principal components block to yield a column of scores. Correlations were then calculated between these scores and superimposed coordinate values.

To provide an intuitive measure of effect strength, a heuristic discriminant function analysis (DFA) was conducted to determine the percentage of specimens that could be correctly classified to the population of origin based on body shape. To facilitate the DFA, the effects of “sex” and “allometry” were removed first by using the residuals of a preparatory MANCOVA. In this MANCOVA, the 24 principal components were used as dependent variables, “centroid size” as a covariate, and “sex” as an independent variable. All statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL, USA).

Results

A total of 245 individuals from the Cueva del Azufre were analyzed. Besides significant allometric effects ($F_{24,213}=19.21$, $P<0.001$, $\eta_p^2=0.684$) and a pronounced sex difference ($F_{24,213}=111.30$, $P<0.001$, $\eta_p^2=0.926$), we found significant body shape variation among individuals from different cave chambers ($F_{72,645}=6.80$, $P<0.001$, $\eta_p^2=0.431$). There was also a significant interaction between sex and cave chamber, however, this effect explained the smallest proportion of variance ($F_{72,645}=2.72$, $P<0.001$, $\eta_p^2=0.239$).

The primary difference between sexes was in the position of the anal fin. In males, the anal fin is modified into a copulatory organ (the gonopodium, characteristic of the subfamily Poeciliinae), which is typically located more anterior than the female anal fin (Rosen and Gordon 1953; Rosen and Bailey 1963). A qualitative inspection of the Cartesian transformation grids (Fig. 2) indicates that fish from

different cave chambers varied predominantly in eye and head size, whereby fish from the innermost and non-sulfidic chamber XIII exhibit the smallest eyes and heads. Quantitatively, the variation in eye and head size is supported by the correlations between superimposed landmarks and the shape gradient among cave chambers as coordinates defining the head and eye size exhibit the largest correlation coefficients (Table 1).

The discriminant function analysis indicated that fish from chamber XIII are most distinct, and fish from chamber II, V, and X are more similar to each other. Over 76 % of the specimens (compared to the expected 25% under a null hypothesis of no pattern) could be assigned to the cave chamber of origin based on morphometric data (Table 2, Fig. 3). Only three out of 57 misclassified individuals belonged to chamber XIII; all other cases were misclassifications among chambers II, V, and X.

Discussion

Poecilia mexicana from different cave chambers within the Cueva del Azufre differ significantly in body shape despite the spatial proximity. As in previous studies on these fish, we found a gradual reduction of eye size from the front chambers that receive some dim light to the rear chambers that are perpetually dark. Simultaneously, we also found a reduction in head size, which was most prominent in the innermost and non-sulfidic cave chamber XIII. Variation in head size has been shown to be correlated with the size of the gills (*Barbus neumayeri*—Langerhans et al. 2007, *P. mexicana*—Tobler et al. 2008a). In sulfidic water, oxygen is scarce, and large gills facilitate efficient oxygen acquisition crucial for survival (Plath et al. 2007b; Tobler et al. 2008a). Increased head size in cave organisms has also been hypothesized to be consequential to natural selection for increased non-visual sensory capabilities (Poulson 1963; Langecker 2000); our data, however, is not consistent with this idea as fish from the innermost cave chamber XIII have the smallest heads. Unlike Gordon and Rosen (1962), we also could not find significant differences in caudal peduncle proportions within the cave. Overall, we documented significant morphological variation among cave chambers that parallels trait divergence among populations living in

Table 1 Correlation coefficients of superimposed landmark coordinates with the shape gradients defined by the independent variables used in the MANCOVA (see text). Correlations $\geq |0.40|$ are given in bold

	Cave chamber	Sex	Centroid size	Cave chamber \times sex
X1	0.17	0.23	-0.17	0.09
Y1	-0.51	-0.18	-0.52	0.20
X2	0.18	0.47	-0.09	-0.08
Y2	0.15	-0.25	-0.07	0.07
X3	-0.07	0.50	0.07	-0.17
Y3	0.12	-0.01	0.39	0.00
X4	0.33	0.08	0.07	-0.39
Y4	0.03	0.19	0.13	0.19
X5	0.07	0.58	0.52	-0.37
Y5	0.06	0.05	-0.27	0.31
X6	0.08	0.49	-0.09	-0.07
Y6	0.00	0.47	-0.10	0.00
X7	0.04	0.50	-0.21	-0.05
Y7	0.08	0.55	-0.28	-0.33
X8	-0.05	-0.63	0.14	0.15
Y8	-0.07	-0.10	0.17	-0.27
X9	-0.03	-0.63	0.07	0.15
Y9	-0.03	-0.16	0.14	-0.30
X10	-0.12	-0.63	-0.04	0.20
Y10	-0.07	-0.21	0.15	0.02
X11	-0.18	0.23	-0.03	0.00
Y11	0.44	-0.09	0.21	-0.02
X12	-0.45	0.16	-0.18	0.22
Y12	-0.08	-0.23	0.24	0.06
X13	0.07	0.61	-0.20	-0.27
Y13	-0.12	-0.37	0.32	0.23
X14	-0.10	0.29	-0.53	0.02
Y14	0.17	-0.27	-0.10	0.08
X15	0.41	0.51	0.38	-0.15
Y15	0.13	-0.24	-0.04	0.06

different habitat types; variation in eye size appears to be related to the presence and absence of light and variation in head size to the presence and absence of H₂S.

Interestingly, the variation in body morphology among cave chambers cannot be explained by the presence of physical barriers, since—apart from the small waterfall between chambers X and XIII—there are none. Consequently, genetic drift alone is an unlikely explanation for the persistence of the morphological gradient. This is in strong contrast with the few other cavefishes, where studies among populations of the same species are available, and morphological differentiation appears to be tied to

physical barriers to migration. For example, populations of the cave-dwelling armored catfish (*Ancistrus cryptophthalmus*) differ in body shape, but populations are isolated topographically (Reis et al. 2006). Similarly, variation in the degree of eye and pigmentation reduction occurs among populations of cave-dwelling Mexican tetras (*Astyanax mexicanus*; Mitchell et al. 1977); however, different populations of this species have colonized caves independently and evolved in isolation (Strecker 2003).

Two not mutually exclusive mechanisms could explain the observed pattern of body shape differences among *P. mexicana* from different cave cham-

Table 2 Canonical discriminant function analysis using body shape data based on geometric morphometrics. The table lists standardized canonical discriminant function coefficients, canonical correlation, eigenvalue, percentage of variance explained, χ^2 -value, degrees of freedom (*Df*) as well as the significance value (*P*) for each of the three discriminant functions

	Function 1	Function 2	Function 3
PCA axis 1	0.089	0.186	0.262
PCA axis 2	-0.590	-0.101	0.633
PCA axis 3	0.202	-0.168	0.013
PCA axis 4	-0.027	0.222	-0.080
PCA axis 5	0.594	-0.155	0.045
PCA axis 6	-0.253	0.402	0.143
PCA axis 7	-0.249	-0.051	-0.080
PCA axis 8	-0.321	-0.188	0.160
PCA axis 9	-0.053	0.087	0.132
PCA axis 10	0.115	-0.310	0.080
PCA axis 11	0.008	-0.026	-0.173
PCA axis 12	-0.218	0.476	0.151
PCA axis 13	-0.218	0.300	-0.068
PCA axis 14	-0.071	-0.458	-0.175
PCA axis 15	0.267	0.454	-0.119
PCA axis 16	-0.090	-0.059	-0.006
PCA axis 17	0.148	-0.240	-0.211
PCA axis 18	0.442	-0.177	0.546
PCA axis 19	0.081	0.209	0.187
PCA axis 20	0.142	0.299	0.054
PCA axis 21	0.198	0.199	0.313
PCA axis 22	0.076	-0.359	0.096
PCA axis 23	-0.055	0.012	0.180
PCA axis 24	0.585	0.215	0.524
Canonical correlation	0.767	0.632	0.498
Eigenvalue	1.427	0.665	0.330
% variance	58.9	27.4	13.6
χ^2	386.737	182.763	65.558
<i>Df</i>	72	46	22
<i>P</i>	<0.001	<0.001	<0.001

bers. Fish may be locally adapted to the particular environmental conditions of a given cave chamber (e.g., different levels of light and/or H_2S concentrations). The extremely low spatial scale (chamber X and XIII are only a few meters apart) may make this scenario look improbable, but fish among some different cave chambers are genetically differentiated despite the spatial proximity (F_{ST} -values based on

microsatellites ranged from 0.022 to 0.108, F_{ST} -values based on cytochrome *b* sequences from 0.444 to 0.594: Plath et al. 2007a; Tobler et al. 2008a). Local adaptation is possible even on small spatial scales with extensive migration if divergent selection is sufficiently strong (Jimenez-Ambriz et al. 2006; Hays 2007; Manier et al. 2007). Furthermore, another study investigating within-cave trait variation found heritable trait divergence; *P. mexicana* from chamber V, which receives dim light through skylights, exhibit opsin gene expression similar to conspecifics from surface populations (despite the reduced eye size), whereas expression of the same genes is reduced in fish from the completely dark chamber X. These differences persist over multiple generations if fish are bred in the laboratory even when kept in daylight (M. Tobler, S. Coleman, B. Perkins and G. Rosenthal, unpublished data).

Alternatively, differences in body shape among cave chambers may be phenotypically plastic (West-Eberhard 1989; Pigliucci 2001). Phenotypic plasticity has previously been hypothesized to play an important role in the phenotypic differentiation of cave-fishes and other cavernicolous organisms (Banister 1984; Cooper et al. 2001; also see Romero and Green 2005 for a review). Variation in body shape of *P. mexicana* has also been found to have a plastic component when comparing fish from cave and

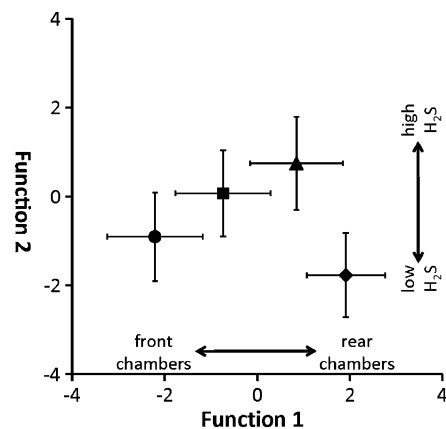


Fig. 3 Canonical discriminant function plot (group centroids \pm standard deviations for the first two discriminant functions) for the analysis presented in Table 1. Symbols represent individuals from cave chamber II (●), V (■), X (▲), and XIII (◆). Along the axis of function 1, populations assemble in an order from the front of the cave to its back; along the axis of function 2, they assemble from low to high H_2S concentrations

surface habitats (Tobler et al. 2008a). However, plastic components predominantly included variation in body height and caudal peduncle proportions and not head or eye size. To distinguish between the two mechanisms, future studies will not only have to estimate the heritability of body shape and its reaction norm across different environments, but differences in body shape have to be shown to affect fitness under the specific environmental conditions.

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