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Pigment cell retention in cavernicolous populations of *Poecilia mexicana* (Poeciliidae)

Beibehaltung von Pigmentzellen in höhlenbewohnenden Populationen von *Poecilia mexicana* (Poeciliidae)

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Summary: The Atlantic molly (*Poecilia mexicana*) is a small livebearing fish that has colonized two cave systems in the southern state of Tabasco, Mexico. Unlike many obligate cave-dwellers (i.e. troglobites) all cave *P. mexicana* retain some pigmentation, as well as a functional visual system. In the Cueva del Azufre the fish occupy habitats (i.e. cave chambers) that differ along a gradient of hydrogen sulfide (H_2S) concentration, as well as a patchwork of light exposure due to several large skylights. While the relationship between eye size and opsin expression with cave distance has been explored, the extent of differences in pigmentation has not yet been quantitatively evaluated. In this study we compared pigment cell (melanophore and xanthophore) count in wild-caught fish from one surface population (Arroyo Bonita) and two cave populations: chambers V (featuring a skylight) and chamber X (exists in perpetual darkness) of the Cueva del Azufre over a 120 day period. Surface populations had significantly higher total numbers of pigment cells than both cave populations, which did not differ significantly from each other. We speculate that skylights in the Cueva del Azufre, paired with a recent evolutionary origin of the cave population and genetic homogenization, have allowed for trait maintenance in cavernicolous *P. mexicana*.

Keywords: Cave fish, cave molly, ecological diversification, extremophile, Poeciliidae

Zusammenfassung: Der Mexikokärpfling (*Poecilia mexicana*) ist ein kleiner, lebendgebärender Fisch, der im Süden Mexikos (Tabasco) zwei Höhlen besiedelt hat. Anders als viele andere obligate Höhlenbewohner sind diese Höhlenfische noch pigmentiert und besitzen funktionsfähige Augen. In der Cueva del Azufre besiedeln die Fische verschiedene Höhlenkammern, die sich in der Konzentration von toxischem Schwefelwasserstoff (H_2S) unterscheiden sowie in der Menge an vorhandenem Licht, das durch verschiedene Deckeneinbrüche in die Höhle eindringt. Während die Beziehung zwischen Höhlenkammer und Augengröße und Opsin-Expression gut verstanden ist, sind die Zusammenhänge mit dem Grad der Pigmentierung noch nicht untersucht worden. In dieser Studie vergleichen wir die Zahlen von Pigmentzellen (Melanophoren und Xanthophoren) bei Fischen einer Oberflächenpopulation (Arroyo Bonita) und zweier Höhlenpopulationen: Kammer V (mit einem Deckeneinbruch) und Kammer X (in vollständiger Dunkelheit) der Cueva del Azufre für 120 Tage. Die Tiere der Oberflächenpopulation hatten signifikant mehr Pigmentzellen als beide Höhlenpopulationen, welche sich nicht signifikant voneinander unterschieden. Wir spekulieren, dass die Deckeneinbrüche in der Cueva del Azufre, geringes evolutionäres Alter und genetische Durchmischung in der Höhle die Pigmentierung der Höhlentiere erhalten haben.

Schlüsselwörter: Höhlenfisch, Höhlenmolly, ökologische Artbildung, extremophil, Poeciliidae

1. Introduction

Troglobitic organisms, i.e. organisms that permanently reside in subterranean habitats and can complete their full life cycle in perpetual darkness, have long-served as evolutionary models in the study of trait regression and trait loss (Culver et al. 1995, Aden 2005). Many cavernicolous organisms exhibit regression of eye size and eye functionality (or even complete eye loss) and loss of pigmentation (i.e. albinism) (Culver et al. 1995, Aden 2005, Culver & Pipan 2009; Romero, 2009).

Furthermore, populations can also vary in the degree of variability associated with troglomorphic traits: Some cavernicolous fishes have apparently lost the ability to re-attain surface pigmentation phenotypes completely, while others recover the epigean pigmentation when exposed to light for prolonged periods of time (all authors, pers. observation; Espinasa & Borowsky 2000, Wilkens, 2001, Romero & Green 2005, Jeffery, 2009). For example, some seemingly albino cave fishes will develop grayish coloration (i.e. increased melanin synthesis) when exposed to light (Culver et al. 1995, Espinasa & Borowsky 2000, Wilkens 2001). In cave environments, secondary light exposure is most often the result of skylights (holes in the cave ceiling, often the result of collapse) (Culver et al. 1995, Espinasa & Borowsky 2000), and such skylights may have allowed for the reacquisition of epigean phenotypes in populations of *Astyanax mexicanus* (Espinasa & Borowsky 2000) and *Gammarus minus* amphipods (Culver et al. 1995).

One model to study pigment evolution in cave fishes is the Atlantic molly (*Poecilia mexicana*; Steindacher, 1863), a small live-bearing fish (Poeciliidae), inhabiting lotic and lentic systems on the gulf coast of Central America, from northern Mexico to Costa Rica (Miller 2005). While the majority of *P. mexicana* populations are epigean, *P. mexicana* has also colonized two known caves in the state of Tabasco (southern Mexico): the Cueva del Azufre (aka. Cueva de Villa Luz or Cueva de las Sardinas) and the Cueva Luna Azufre (Gordon & Rosen 1962; Tobler et al. 2008b). Unlike many other caves, the Cueva del Azufre is not a completely dark environment: a patchwork of more than 20 skylights permeates the cave (Parzefall 2001). One of the most prominent skylights in the Cueva del Azufre allows light of up to 320 lux into cave chamber V (Fig. 1; Gordon & Rosen

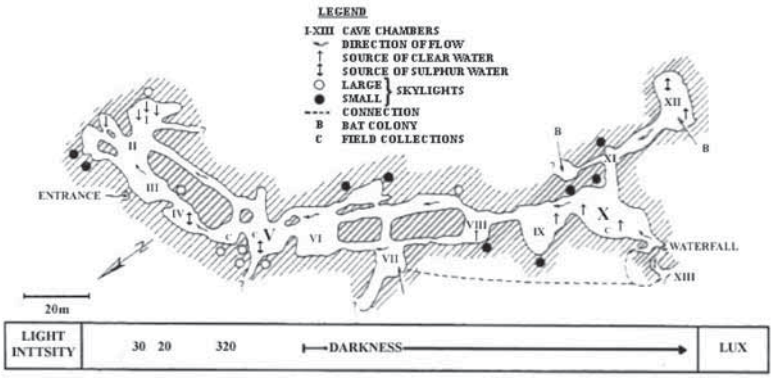

Fig. 1: Map of the Cueva del Azufre near Tapijulapa, Tabasco, Mexico. Skylights are indicated for each cave chamber as well as light intensity measured in a limited number of chambers. Map was modified for clarity from Parzefall (2001, drawn by Monika Hänel) and also marks the location of field collections for this study in cave chambers V and X.

Abb. 1: Karte der Cueva del Azufre bei Tapijulapa, Tabasco, Mexico. Gezeigt werden Deckeneinbrüche und Lichtintensitäten für die verschiedenen Höhlenkammern. Die Karte basiert auf einer Zeichnung aus Parzefall (2001, gezeichnet von Monika Hänel) und zeigt die Herkunft der Tiere für diese Studie aus den Kammern V und X.

1962, Hose & Pisarowicz 1999; Parzefall 2001). Although some skylights exist beyond cave chamber V, very little light penetrates into the deeper parts of the cave, essentially leaving these chambers in perpetual darkness (0.005 lux in cave chamber X and XIII; Parzefall 2001).

Although field-caught cavernicolous *P. mexicana* appear completely void of pigment, most cave-dwelling *P. mexicana* visually appear to develop grayish pigmentation typical of surface populations when housed in illuminated laboratory conditions for prolonged periods of time (Parzefall 2001; all authors, pers. observation). Species inhabiting a cave system are usually considered a single population and therefore, degrees of trait loss are often compared between caves and epigean counterparts and not between different populations from within a single cave. In the Cueva del Azufre gene flow is reduced and only occurs from "inside-to-outside" of the cave (Tobler et al. 2008a; Tobler et al. 2009b; R. Riesch & I. Schlupp, unpubl. data). However, there also appears to be selection against migrants between cave chambers (Tobler et al. 2009a), resulting in small-scale differentiation even within the cave (Plath et al. 2007a; Tobler et al. 2008a, Plath et al. 2010). Despite flood events that have been found to temporarily homogenize genetic differentiation among populations within a habitat type (i.e. within the cave or surface) (Plath et al. 2010), various studies have noted differences in behavior (Parzefall, 2001; Plath et al. 2007a; Tobler et al. 2009a), eye size (Parzefall 2001), opsin gene expression (Tobler et al. 2009a), morphology (Tobler 2008a, Tobler et al. 2008b, Fontanier & Tobler 2009) and life history (Riesch et al. 2010, 2011*a*) between cave and surface populations that often follow a gradient within the cave with more extreme phenotypes in the innermost chambers.

In the present study we hypothesize that a relatively recent colonization, paired with exposure to light through skylights and occasional gene flow as a result of extreme flood events, may be responsible for the persistence of pigment cells in *P. mexicana* from the Cueva del Azufre. In order to address this, we compared pigment cell response between surface fishes (Arroyo Bonita) and two populations from the Cueva del Azufre (chambers V and X) randomly assigned to a 12:12 hr light:dark cycle or permanent darkness over a 120 day period. These three populations enabled us to investigate pigment cell count across the natural gradient of light exposure from full exposure to sunlight to permanent darkness (Parzefall 2001), and we chose to assess melanophore (black) and xanthophore (orange) chromatophores in all populations (Jeffery 2006).

We asked: (1) Are there differences between melanophore, xanthophore or total pigment cell count/size between cave and surface populations? (2) Does pigment cell count or size change over time in *P. mexicana* populations when exposed to light or dark conditions, and (3) could skylight exposure explain patterns of pigment cell count between cavernicolous populations of *P. mexicana* from a *single* cave?

Based on previous observations, we predicted differences in chromatophore count between surface and cave forms, as well as between cave populations. More specifically, we expected chromatophore count to decrease over time in surface fish as well as chamber V cave fish when held in a perpetually dark laboratory environment, while chromatophore count of chamber X cave fish should be unaffected. Similarly, we expected chromatophore count to increase in chamber V cave fish when raised under a light:dark cycle, but not to be affected in surface fish and chamber X fish.

2. Material and Methods

2.1. Study system/populations

We collected surface- and cave-dwelling *P. mexicana* females near the village of Tapijulapa, Tabasco, Mexico, in January 2010. We specifically excluded males from the study as we expected a higher degree of pigment display and therefore variability due to sexually-selected ornaments (Endler 1983, 1992). We collected approximately twelve cavernicolous *P. mexicana each* in chambers V and X of the Cueva del Azu-

fre using dip nets (fig. 1) and surface-dwelling *P. mexicana* with a seine from the Arroyo Bonita (17º 25' 37.42" N, 92º 45' 6.98" W), a small fresh-water tributary of the Río Oxolotán. The Arroyo Bonita is approximately two kilometers downstream from where the resurgence outflow of the Cueva del Azufre connects to the Río Oxolotán (Riesch et al. 2010).

2.2. Experimental Design

Upon capture, fishes were kept in coolers with aerated water for transport to the University of Oklahoma in Norman, USA, and light exposure was limited to a few minutes every day when fish were checked for health and water was changed. Once in the laboratory, fishes were kept in a "common garden" set-up in either perpetual darkness (dark room, photosynthetically active radiation (PAR) 1.0±1.0 μmol m^{-2} s^{-1}, mean±SD) or in 12 hour light/12 hour dark cycle of lighting (light room, PAR: 50.0±7.2 μmol m^{-2} s^{-1} during the light cycle). The light intensity in our light room setup could be considered relatively high in comparison to natural conditions in the tropics (Endler 1991, 1993, 2001).

Each fish was individually housed in a 2-gallon aquarium containing small pebbles and a sponge filter. Individual fish were assigned a treatment and tank number through the use of a random number generator, so that we had twelve fish from each population in each treatment. Fish were fed twice daily on an *ad libitum* diet of frozen *Daphnia* and fresh or frozen brine shrimp (*Artemia*). For the purpose of feeding and a general health check, fish in the dark treatment were exposed to red light from a headlamp for a few seconds each day. Water was changed and tanks were cleaned every two weeks. For fish in the dark treatment, water changes were conducted in an unlit room adjacent to the dark room. Although diffuse light entered the room from a small window in the door, we considered the amount and time of exposure negligible (4.7±0.6 μmol m^{-2} s^{-1}).

Initially upon arrival to the laboratory, we allowed all fish to acclimate for approximately two weeks in their respective experimental setup. Once acclimated, the experiment lasted approximately 120 days. Every ten days during the actual experiment one individual fish was randomly selected from each population in each treatment for tissue sampling. For this purpose fish were removed from their tanks and the dorsal half of the caudal fin was clipped (Felice et al. 2008). Fish were sexed, weighed, measured for standard length and subsequently returned to common stocks for their respective population. Fin clips were photographed at a magnification of 1120x using an InSight Spot 2 digital camera (Diagnostic Instruments) mounted to an Olympus SX 7 stereomicroscope using Spot® imaging software and were then stored in 37% formaldehyde, while image files were stored in TIFF format for subsequent analyses.

To obtain pigment cell counts, a 1 mm by 1 mm grid based on standardized and calibrated pixel-to-mm ratios in Spot® imaging software was created in Adobe Photoshop® and overlaid on each image file. Five random 1 mm by 1 mm grid squares that overlapped the fin clip in each image file were selected. Grid squares that did not directly overlap the fin clip on the image

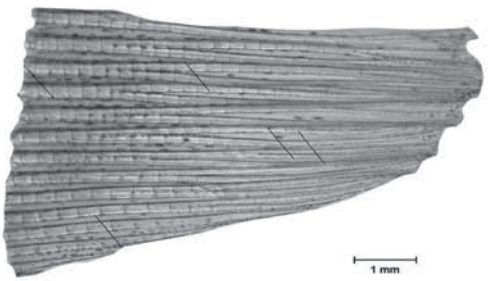

Fig. 2: Transect lines placed randomly on a caudal fin clip image. A 1 mm x 1 mm grid was overlaid on each image and a random number generator was used to select grid cells. Selected cells had a diagonal transect line placed from the top left to bottom right corner of each grid. For further details see text.
Abb. 2: Linien wurden zufällig über ein Foto eines Flossenabschnitts gelegt. Ein 1 mm x 1 mm-Netz wurde über jedes Foto gelegt und mit Hilfe eines Zufallsgenerators wurden die Zellen ausgewählt, die ausgezählt werden sollten. In diese Zellen wurde dann eine Diagonallinie von links oben nach rechts unten gelegt. Weitere Erklärungen s. Text.

were discarded and not considered for analysis. A diagonal transect line was then drawn from the top left corner to the bottom right corner of each grid square in Adobe Photoshop®. The grid layer was then deleted, leaving only the five transect lines on each fin clip image (fig 2). Afterwards, each image was assigned a random number in order to avoid observer bias. Only cells in which the transect line completely overlapped the fin were used. If a selected grid cell contained an air bubble or the fin was folded in some manner, it was excluded and replaced. Melanophores and xanthophores were counted if they contacted a transect line, and were subsequently measured in diameter using Spot software. All counts were conducted by magnifying each transect line with as little image distortion as possible (observer discretion).

As an additional preventative measure against observer bias, the actual measurements of pigment cell numbers for each fin clip were conducted by a student unfamiliar with the experimental treatments administered and hypotheses tested in our study. Melanophores and xanthophores that directly contacted each transect line were counted and measured in diameter for each fin clip. Because pigment cells have highly variable shapes (pers. observation), we decided to measure the approximate cell diameter as the maximum continuous diameter (in millimeters) using the measurement function in Spot software. This provided us with counts for melanophores and xanthophores, as well as total pigment cells (i.e., the sum of melanophores and xanthophores). Over the course of our experiment, three fish matured into males (i.e., developed the male copulatory organ or gonopodium), and therefore, their data were excluded from analysis in order to avoid sex-based bias (i.e., males are usually the more colorful sex in Poeciliidae; Farr 1989). Unexpectedly, chamber X fish in the light treatment experienced relatively high mortality (N=6), with no fish remaining in the treatment past the fifth week. No other populations (in either treatment), experienced excessive or unexpected mortality rates through the course of the experiment.

Since both the pigment cell counts and cell size data for both melanophores and xanthophores were not normally distributed (Shapiro-Wilks test: $p<0.05$ in all cases), we used Kruskal-Wallis tests. Our dependent variables were either melanophore, xanthophore, and total cell counts or size with population (surface vs. chamber V vs. chamber X) as the factor. In order to assess differences in pigment cell type or size between treatments in our experiment we used a Mann Whitney U test with treatment (dark or light) as the factor. Due to the use of multiple comparisons (i.e. cell sizes and cell types), we applied the Bonferroni correction that adjusted our alpha value from $p\leq0.050$ to $p\leq0.025$ in order to avoid Type I error.

Finally, to assess the influence of time in the experimental treatment on pigment cell count, we tracked changes in melanophores, xanthophores and total cells in each population and each experimental treatment over the 120-day time period using several independent linear regressions (cell counts modeled versus time). All statistics were performed in SPSS 17.0.

3. Results

3.1. Pigment cell count (number of cells)

Descriptive statistics regarding cell count for all fish in both dark (fig. 3) and light (fig. 4) experimental treatments can be found in table 1. The Mann Whitney U tests with treatment as a factor revealed non-significant differences in the median number of xanthophores ($U(1)=392.000$; $Z=-0.2131$; $p>0.05$), melanophores ($U(1)= 392.000$; $Z=-0.210$; $p>0.05$) and total number of cells ($U(1)=338.500$; $Z = -0.771$ $p>0.05$). Generally, fish kept in the light treatment developed a similar number of pigment cells as their counterparts in the dark treatment (fig. 5). However, our surface fish developed more xanthophores in the light treatment, and thus more total cells (tab. 1), which may have driven our overall analysis to significance between treatments.

In the separate Kruskal-Wallis H-test on population differences, median number of melanohores ($KW(2)= 23.208$, $p<0.001$),

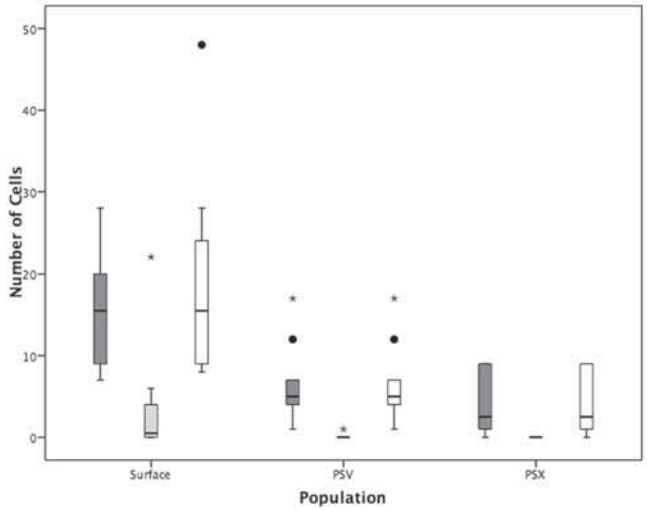

Fig. 3: Median total pigment cell (black), melanophore (grey) and xanthophore (white) count in *P. mexicana* of Arroyo Bonita (Surface) and Cueva del Azufre chambers V (PSV) and X (PSX) in the experimental dark treatment. Whiskers (error terms) represent 95% confidence intervals. Although data points exceeding the range of the whiskers may represent outliers, we included all data points due to a limited sample size.
Abb. 3: Mediane und 95 %-Konfidenzintervale für totale Pigmentzellen (schwarz), Melanophoren (grau) und Xanthophoren (weiß) für *P. mexicana* vom Arroyo Bonita (Oberfläche) und Cueva del Azufre Höhlenkammern V (PSV) und X (PSX) in Dunkelhaltung. Wegen der kleinen Stichprobengröße wurden auch Extremwerte für die Datenanalyse genutzt.

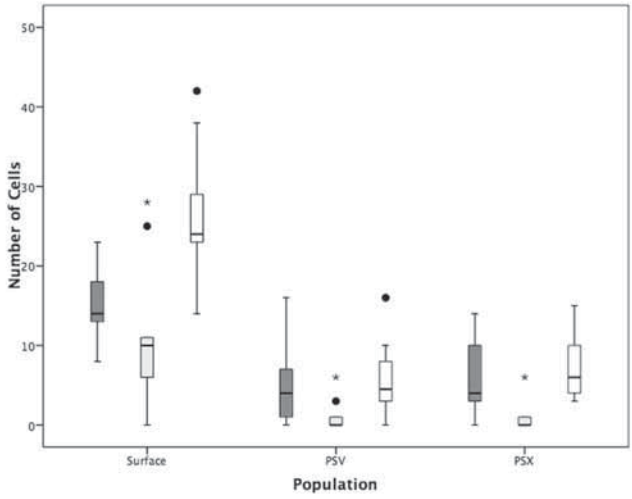

Fig. 4: Median total pigment cell (black), melanophore (grey) and xanthophore (white) count in *P. mexicana* of Arroyo Bonita (Surface) and Cueva del Azufre chambers V (PSV) and X (PSX) in the experimental light treatment. Whiskers (error terms) represent 95% confidence intervals. Although data points exceeding the range of the whiskers may represent outliers, we included all data points due to a limited sample size.
Abb. 4: Mediane und 95 %-Konfidenzintervale für totale Pigmentzellen (schwarz), Melanophoren (grau) und Xanthophoren (weiß) für *P. mexicana* vom Arroyo Bonita (Oberfläche) und Cueva del Azufre Höhlenkammern V (PSV) und X (PSX) in Lichthaltung. Wegen der kleinen Stichprobengröße wurden auch Extremwerte für die Datenanalyse genutzt.

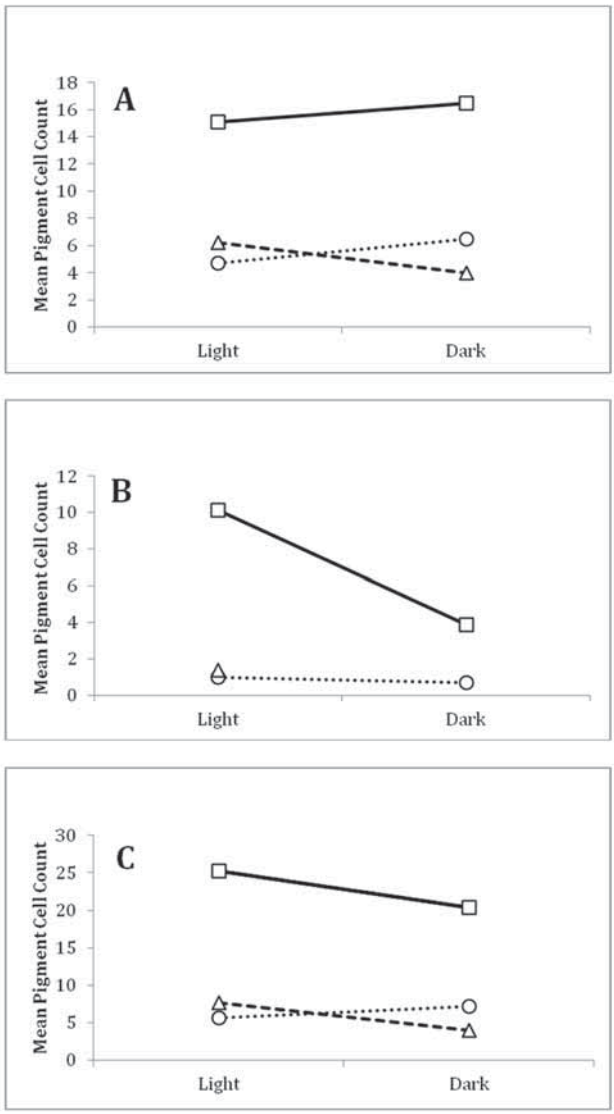

Fig. 5: Comparison of mean melanophore (A), xanthophore (B) and total cells (C) in *Poecilia mexicana* populations from the Arroyo Bonita (surface) and chamber V and chamber X of the Cueva del Azufre. After a two week acclimatization period, fish were kept under light or dark conditions for approximately 100 days and pigment cell counts were obtained from a caudal fin clip from a fish from each population in each treatment. Solid lines represent Arroyo Bonita, dashed lines represent chamber X, and dotted lines represent chamber V. Random mortality led to the absence of data for xanthophores and chamber X.

Abb. 5: Vergleich der durchschnittlichen Zellzahl (A = Melanophoren; B = Xanthophoren; C = alle Zellen) für *Poecilia mexicana* von Arroyo Bonita (Oberflächenpopulation) und Höhlenkammern V und X. Nach einer Eingewöhnungszeit von zwei Wochen wurden die Tiere für ca. 100 Tage in Licht (Light) oder Dunkelheit (Dark) gehalten. Pigmentzellen wurden anhand eines Flossenstücks von der Schwanzflosse von einem Fisch pro Population und Behandlung bestimmt. Durchgezogene Linien stehen für Arroyo Bonita, gestrichelte Linien stehen für Kammer X, und gepunktete Linien stehen für Kammer V. Zufällige Mortalität ist für die fehlenden Daten bei Kammer X und Xanthophoren verantwortlich.

xanthophores (KW(2)= 12.131, p<0.010) and total cells (KW(2)= 26.534, p<0.001) were significantly different between populations. Pairwise comparisons utilized post-hoc revealed highly significant differences between surface and chamber V fish in median melanophores (p<0.001), xanthophores (p<0.010), and total cells (p<0.001). Additionally, surface fish differed from chamber X fish in the median number of melanophores (p<0.001), xanthophores (p<0.05), and total cells (p<0.001). Surface fish exhibited more melanophores, xanthophores and total cells on average than both chamber V and chamber X fish from the Cueva del Azufre (tab. 1). However, cave fish from chamber V and X did not differ significantly in median melanophores (p>0.05), xanthophores (p>0.05), and total cells (p>0.05).

Tab. 1: Descriptive statistics for pigment cell counts in caudal fin clips of surface (Arroyo Bonita), cave chamber V and cave chamber X fishes (Cueva del Azufre) housed in dark and light common-garden setups. Pigment cells were counted along five randomly selected transect lines in each fin clip. Values represent medians with the interquartile range in parentheses.

Tab. 1: Beschreibende Statistik der Pigmentzellzahlen in Schwanzflossenabschnitten von Oberflächentieren (Arroyo Bonita), Kammer V und Kammer X der Höhle Cueva del Azufre aus Dunkel- und Lichthaltung. Die Pigmentzellen wurden entlang von fünf zufällig ausgewählten Linien in jedem Flossenabschnitt gezählt. Gezeigt werden Mediane und Interquartilabstände (in Klammern).

Treatment	Population	N (Surviving)	Melanophores	Xanthophores	Total Cells
Dark	Surface	11	16.00 (14.00)	1.00 (5.00)	16.00 (11.00)
	Chamber V	10	5.00 (5.50)	0.00 (0.25)	5.00 (4.00)
	Chamber X	10	2.50 (8.25)	0.00 (0.00)	2.50 (8.25)
Light	Surface	10	14.00 (6.00)	10.00 (18.00)	24.00 (14.50)
	Chamber V	12	4.00 (6.25)	0.00 (1.50)	4.50 (6.00)
	Chamber X	6	4.00 (5.50)	0.00 (3.50)	6.00 (9.00)

Tab. 2: Descriptive statistics for linear regressions performed on pigment cell counts (melanophores, xanthophores and total cells) over the course of time (100 day experiment) in *Poecilia mexicana* from the Arroyo Bonita (surface) and from chambers V and X of the Cueva del Azufre. Pigment cell data was obtained from single point measurements (individual fish) at each time point (every 10 days). In our analysis, we did not find any xanthophores in Chamber X fish kept in our dark treatment, therefore, there are no values from the statistical analysis. Significant P-values are listed in bold.

Tab. 2: Hier fehlt der deutsche Text.

Treatment	Population	Melanophores			Xanthophores			Total Cells		
		r^2	Slope	*p*	r^2	Slope	*p*	r^2	Slope	*p*
Dark	Surface	0.204	-1.05	0.222	0.021	-0.32	0.710	0.130	-1.37	0.342
	Chamber V	0.423	-1.22	**0.042**	0.002	-0.03	0.901	0.364	-1.25	0.065
	Chamber X	0.672	-1.17	**0.046**	-	-	-	0.672	-1.17	**0.046**
Light	Surface	0.339	-0.28	0.636	0.108	1.25	0.388	0.067	0.97	0.502
	Chamber V	0.336	-0.90	0.079	0.245	0.33	0.145	0.134	-0.58	0.298
	Chamber X	0.411	-2.30	0.244	0.061	-0.10	0.923	0.593	-2.40	0.128

3.2. Pigment cell count (cell size)

We could not detect significant differences between melanophore and xanthophore size in neither the Kruskal-Wallis H-tests (melanophores: KW(2)=1.402; p>0.025; xanthophores: KW(3)=0.212; p>0.05) on population differences nor the Mann Whitney U test on treatment differences (melanophores: U(1)=23927.50; Z= -1.101; p>0.05; xanthophores: U(1)=1948.500; Z=-0.460 ; p>0.05).

3.3. Time-series analysis of pigment cell count

Overall, linear regressions indicated non-significant declines over time through the duration of our experiment in melanophores, xanthophores, and total cells for the majority of the *P. mexicana* populations in both dark and light experimental conditions (tab. 2). In fact, the only significant decreases over the 120-day period were uncovered for melanophore counts of both cave populations (chambers V and X; tab. 2). Pigment cell increases (albeit non-significant), however, were only found for xanthophores in surface fish (here also affecting total cell count) and chamber V fish kept in light over the course of the experiment (tab. 2).

4. Discussion

In our experiment, cavernicolous *P. mexicana* had fewer melanophores and xanthophores than their surface dwelling counterparts and it appears as though the degree of pigmentation loss may be slightly correlated with the gradient of light exposure in the Cueva del Azufre that occurs due to a patchwork of skylights (i.e. chamber X fish lacked xanthophores, which are still present in chamber V fish). However, surface and cave fish did not differ in chromatophore size and we did not notice a difference in response to light between the surface and cave individuals.

Our linear regression analyses revealed that most fish, regardless of population, exhibited negative trends for the number of pigment cells becoming detectable over the course of our experiment in both the dark and light treatments. While decreases in pigment cells were expected for fish in our dark treatment (three trends significant, tab. 2), the negative pigment development trends for fish in our light treatment (especially melanophores) was surprising in comparison to previous work on pigment development in teleosts (reviewed by Leclercq et al. 2010). It seems that formation and loss pigment cells during ontogeny is very complex and variable. Our study ignored any potential role of UV in the formation of pigmentation as well as underlying hormonal mechanisms (Leclercq et al. 2010).

Why would melanophore numbers decrease over time in the light treatment for both surface and cavernicolous populations of *P. mexicana* in our experiment? The light room was lit by fluorescent light bulbs, which differ strongly from natural sunlight (e.g., they typically emit lower levels of UV light in comparison to natural sunlight (Endler 1993; Endler 2001). Therefore, this lower exposure to UV light, in particular, could have negatively influenced melanophore development in *P. mexicana* housed in our experimental conditions. Interestingly, the pattern was reversed for xanthophore development in our experiment, which suggests that lower levels of UV light intensity may trigger their development relative to the development of melanophores. However, previous work in other teleost systems has shown xanthophore aggregation under high levels of UV light (most prominent at 400-420 nm) (Oshima et al. 1998, Oshima 2001, Leclercq et al. 2010). This problem needs to be addressed in future research.

4.1. Maintenance of pigmented phenotypes

Could a constant influx of surface fish explain the sustained surface phenotypes in this cave population? Population genetics analyses did not detect any gene flow from the surrounding surface habitats into the cave (Plath et al. 2007a; Tobler et al. 2008a, 2009b), with the exception of immediately after a catastrophic flood (Plath et al. 2010). A much larger genetic signature, however, was found for increased homogeni-

zation between different cave chambers as a result of this flooding, which temporarily led to the breakdown of the strong genetic structure experienced within the cave prior to flooding (Plath et al. 2010). It is therefore possible that periodic floods constantly reintroduce eye and pigmentation genes into the distal-most cave chambers, slowing regressive processes within these populations.

It is interesting to note that only one chamber X fish was found to express xanthophores (tab. 1). The lack of xanthophores in many cave chamber X fish is somewhat surprising in that xanthophores are derived from the same cells as melanophores and disperse in the same fashion (Erickson 1993; Felice et al. 2008). Xanthophores have been retained in other fishes, like the Cave tetra, *Astyanax*, even in the absence of melanophores (Wilkens 1988, Blanchard et al. 1991, McCauley et al., 2004, Jeffery 2009) and have been suggested to be important in orienting melanophore patterning or striping (Parichy 2003), although cell-cell interactions may play a role (Kelsh 2004). Xanthophores typically contain pteridines that are synthesized and/or carotenoids that need to be acquired through diet, because animals cannot synthesize carotenoids de novo (Goodwin 1986, Blanchard et al. 1991). While carotenoids have been found in cave systems previously (Beatty 1941), the amount of available carotenoids and their acquisition by *P. mexicana* in the Cueva del Azufre remain unknown. Nonetheless, a recent study on embryo development in cave- and surface-dwelling *P. mexicana* suggests that chamber V and X fish do in fact have to cope with limited availability of carotenoids (Riesch et al. 2011c). It thus appears as though the retention of xanthophores in some cavernicolous populations of *P. mexicana* may be for purposes that are poorly understood, and surely warrants further consideration.

Surprisingly, we did not find any differences in chromatophore size between surface- and cave-dwelling *P. mexicana*. This is somewhat unexpected, but could potentially be explained by a stress response. If fishes were stressed when fin clips were taken, melanin regulation and subsequent chromatophore display (i.e., size) could have been negatively affected in our visual survey of fin clips. In other words, it is possible that cave fish do in fact have smaller chromatophores than surface fish; however stress-induced shrinkage may have led to a decrease of cell display that converges on a common minimum cell size, effectively rendering an existing size difference undetectable by our survey method.

4.2. Conclusions and Future Directions

Unlike most other troglobites, cave-dwelling *P. mexicana* have not completely lost their pigment cells. The level of reduction likely indicates a relatively recent colonization of the cave system and/or continued maintenance of pigmentation due to light exposure, paired with genetic homogenization through flood events (Plath et al. 2010). The geological history of the Cueva del Azufre as well as the colonization date of the cave by *P. mexicana* relative to cave formation remain largely unknown. Two scenarios are plausible: (1) *P. mexicana* colonized the Cueva del Azufre and little geological change has occurred since, or (2) *P. mexicana* colonized the Cueva del Azufre as a "true" cave environment and skylights formed after colonization. In the former scenario, presence of skylights may select for pigmented and eyed phenotypes. Alternatively, in the later case, there may be degrees of eye and pigment loss that are masked by the reacquisition of surface-like phenotypes in populations inhabiting secondarily lit cave chambers. Unfortunately, the pigmentation data from the present study did not provide us with enough clues as to which scenario appears to be more realistic. Future research will therefore have to investigate this further.

Abiotic gradients are becoming important topics of study in evolution and divergence, and certainly require further investigation in numerous taxa. Ecological trait divergence in cavernicolous *P. mexicana* may be due to the complex suite of stresses (degree of light exposure, hydrogen sulfide and hypoxia) that the Cueva del Azufre imposes on the fish (e.g. Riesch et al. 2011b). Future work should con-

sider elucidating the individual and collective effects of darkness and hydrogen sulfide on the ecology and evolution of trait divergence in *P. mexicana*. A larger-scale study might apply the same framework, but with a higher degree of replication and increased temporal component as the expression of melanin relative to environmental conditions is known to be plastic.

Finally, future assessments should consider using L-DOPA assays in order to better characterize the possibility that pigment cell precursors with melanin synthesis potential are present in cave *P. mexicana,* as they are in *Astyanax* cavefish (McCauley et al. 2004). Although previous studies have used clips of the caudal fins of cave fish to assess pigment development (Felice et al. 2008), assessing pigment development in other fins, or scales in a single fish over time may be more insightful with regards to the differing visual appearance of *P. mexicana* in our two treatments. Fine-scale population comparisons in eye size, pigment cell count and light exposure may yield more conclusive results on the significance of skylights on trait expression. Additionally, fine-scale population genetics comparing cave chambers or between fishes inhabiting lit and unlit portions of a single chamber should be explored in order to better understand trait maintenance in cavernicolous *P. mexicana*, especially populations inhabiting perpetually dark chambers.

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Literature

Aden, E. 2005. Adaptations to Darkness, pp. 1-3. In: Encyclopedia of Caves (D.C. Culver & W.B. White, eds). Elsevier Academic Press, London.

Beatty, R.A. 1941. The pigmentation of cavernicolous animals. II. Carotenoid pigments in the cave environment. Journal of Experimental Biology 18, 144-152.

Blanchard, P.D., R.A. Angus, R.L.Morrison, S.K. Frost-Mason, & J.H. Sheetz. 1991. Pigments and ultrastructure of pigment cells in xanthic sailfin mollies (*Poecilia latipinna*). Pigment Cell Research 4, 240-246.

Christiansen, K.A. 1992. Cave life in the light of modern evolutionary theory, pp. 453-478. In: The natural history of biospeleology (A. Camacho, ed.). Madrid Museo Nacional de Ciencias Naturales, Madrid.

Culver, D.C, Kane, T.C., & D.W. Fong.1995. Adaptation and natural selection in caves: the evolution of *Gammarus minus.* Harvard University Press, Cambridge.

Culver, D.C. & T. Pipan. 2009. The biology of caves and other subterranean habitats. Oxford University Press, Oxford.

Endler, J.A. 1983. Natural and sexual selection on color patterns in poeciliid fishes. Environmental Biology of Fishes 9, 173-190.

Endler, J.A. 1992. Signals, signal conditions and the direction of evolution. The American Naturalist 139, 125-153.

Endler, J.A. 1991. Variation in the appearance of guppy color patterns to guppies and their predators under visual conditions. Vision Research 31, 587-608.

Endler, J.A. 1993. The color of light in forests and its implications. Ecological Monographs 63, 1-27.

Endler, J.A., Basolo, A., Glowacki, S., & J. Zerr. 2001. Variation in response to artificial selection for light sensitivity in guppies (*Poecilia reticulata*). The American Naturalist 158, 36-48.

Erickson, C.A. 1993. From the crest to the periphery: control of pigment cell migration and lineage segregation. Pigment Cell Research 6, 336-347.

Espinasa, L., & R. Borowsky. 2000. Eyed cave fish in a karst window. Journal of Cave and Karst Studies 62, 180-183.

Farr, J.A. 1989. Sexual selection and secondary sexual differentiation in poeciliids: determinants of male mating success and the evolution of female choice, pp. 91-123. In: Ecology & Evolu-

tion of Livebearing Fishes (Poeciliidae) (Meffe, G.K., & F.F. Snelson, Jr., eds.). Prentice Hall, Englewood Cliffs.

Felice, V., Aparecida Visconit, M., & E. Trajano. 2008. Mechanisms of pigmentation loss in subterranean fishes. Neotropical Ichthyology 6, 657-662.

Fontanier, M.E., & M. Tobler. 2009. A morphological gradient revisited: cave mollies vary not only in eye size. Environmental Biology of Fishes 86, 285-292.

Goodwin, T.W. 1986. Metabolism, nutrition, and function of carotenoids. Annual Review of Nutrition 6, 273-297.

Gordon, M.S., & D.E. Rosen. 1962. A cavernicolous form of the poeciliid fish *Poecilia sphenops* from Tabasco, Mexico. Copeia 2, 360-368.

Hose, L.D., & J.A. Pisarowicz. 1999. Cueva de Villa Luz, Tabasco, Mexico: Reconnaissance study of an active sulfur spring cave and ecosystem. Journal of Cave and Karst Studies 61, 13-21.

Jeffery, W.R. 2001. Cavefish as a model system in evolutionary developmental biology. Developmental Biology 231, 1-12.

Jeffery, W.R. 2006. Regressive evolution of pigmentation in the cavefish *Astyanax*. Israel Journal of Ecology & Evolution 52, 405-422.

Jeffery, W.R. 2009. Regressive evolution in *Astyanax* cavefish. Annual Reviews Genetics 43, 25-47.

Kelsh, R.N. 2004. Genetics and evolution of pigment patterns in fish. Pigment Cell Research 17, 326-336.

Leclercq, E., Taylor, J.F., & H. Migaud. 2010. Morphological skin colour changes in teleosts. Fish & Fisheries 11, 159-193.

McCauley, D.W., Hixon, E., & W.R. Jeffery. 2004. Evolution of pigment cell regression in the cavefish *Astyanax*: a late step in melanogenesis. Evolution & Development 6, 209-218.

Miller, R.R. 2005. Freshwater fishes of Mexico. University of Chicago Press, Chicago.

Oshima, N., Nakata, E., Ohta, M., & S. Kamagata. 1998. Light-induced pigment aggregation in xanthophores of the medaka, *Oryzias latipies*. Pigment Cell Research 11, 362-367.

Oshima, N. 2001. Direct reception of light by chromatophores of lower vertebrates. Pigment Cell Research 14, 312-319.

Parichy, D.M. 2003. Pigment patterns: fish stripes and spots. Current Biology 13, R947–R950.

Parzefall, J. 2001. A review of morphological and behavioural changes in the cave molly, *Poecilia mexicana*, from Tabasco, Mexico. Environmental Biology of Fishes 62, 263-275.

Plath, M., Körner, K.E., Schlupp, I., & J. Parzefall. 2002. Sex recognition and female preferences of Cave mollies *Poecilia mexicana* in light and darkness. Memoires de Biospeologie 28, 63-67.

Plath, M., Hauswaldt, S., Moll, K., Tobler, M., García de León, J., Schlupp, I., & R. Tiedemann. 2007. Local adaptation and pronounced genetic differentiation in an extremophile fish, *Poecilia mexicana*, inhabiting a Mexican cave with toxic hydrogen sulphide. Molecular Ecology 16, 967-976.

Plath, M., Blum, D., Tiedemann, R., & I. Schlupp. 2008. A visual audience effect in cave fish. Behavior 145, 931-947.

Plath, M., Hermann, B., Schröder, C., Riesch, R., Tobler, M., García de Léon, F.J., Schlupp, I., & R. Tiedemann. 2010. Locally adapted fish populations maintain small-scale genetic differentiation despite pertebation by catastrophic flood event. BMC Evolutionary Biology 10, 256.

Poulson, T.L. 1963. Cave adaptation in amblyopsid fishes. The American Midland Naturalist 70, 257-290.

Protas, M., Conrad, M., Gross, J.B., Tabin, C., & R. Borowsky. 2007. Regressive evolution in the Mexican cave tetra, *Astyanax mexicanus*. Current Biology 17, 452-454.

Riesch, R., Plath, M., & I. Schlupp. 2010. Toxic hydrogen sulfide and dark caves: life-history adaptations in a livebearing fish (*Poecilia mexicana*, Poeciliidae). Ecology 91, 1494-1505.

Riesch, R., Plath, M., & I. Schlupp. 2011a. Toxic hydrogen sulphide and dark caves: pronounced male life-history divergence among locally adapted *Poecilia mexicana* (Poeciliidae). Journal of Evolutionary Biology 24, 596-606.

Riesch, R., Plath, M., & I. Schlupp. 2011b. Speciation in caves: experimental evidence that permanent darkness promotes reproductive isolation. Biology Letters 7, 909-912.

Riesch, R., Schlupp, I., Langerhans, R.B., & M. Plath. 2011c. Shared and unique patterns of embryo development in extremophile poeciliids. PLoS ONE 6, 1-11.

Romero, A. 2009. Cave biology: life in darkness. Cambridge University Press, New York.

Romero, A., & S.M. Green. 2005. The end of regressive evolution: examining and interpreting the evidence from cave fishes. Journal of Fish Biology 67, 3-32.

Tobler, M. 2008. Divergence in trophic ecology characterizes colonization of extreme habitats. Biological Journal of the Linnean Society 95, 517-528.

Tobler, M., DeWitt, T.J., Schlupp, I, García de León, F.J., Herrmann, R., Feulner, P.G.D., Tiedemann, R., & M. Plath. 2008a. Toxic hydrogen sulfide and dark caves: phenotypic and genetic divergence across two abiotic environmental gradients in *Poecilia mexicana*. Evolution 62, 2643-2649.

Tobler, M., Riesch, R., García de León, F.J., Schlupp, I., & M. Plath. 2008b. A new and morphologically distinct population of cavernicolous *Poecilia mexicana* (Poeciliidae: Teleostei). Environmental Biology of Fishes 82, 101-108.

Tobler, M., Coleman, S.W., Perkins, B.D., & G.G. Rosenthal. 2009a. Reduced opsin gene expression in a cave-dwelling fish. Biology Letters 6, 98-101.

Tobler, M., Riesch, R., Tobler, C.M., Schulz-Mirbach, T., & M. Plath. 2009b. Natural and sexual selection against immigrants maintains differentiation among micro-allopatric populations. Journal of Evolutionary Biology 22, 2298-2304.

Trajano, E. 2001. Ecology of subterranean fishes: an overview. Environmental Biology of Fishes 62, 13-160.

Trajano, E., Reis, R.E., & M.E. Bichuette. 2004. *Pimelodella spelaea*, a new cave catfish from Central Brazil, with data on ecology and evolutionary considerations (Siluriformes: Heptapteridae). Copeia 2, 315-325.

Trajano, E., & M.E. Bichuette. 2005. Diversity of subterranean fishes in Brazil, pp. 161-163. In: World Subterranean Biodiversity (Gibert, J., ed.). Proceedings of an International Symposium held on 8-10 December 2004 in Villeurbaine.

Wilkens, H. 1988. Evolution and genetics of epigean and cave *Astyanax fasciatus* (Characidae: Pisces). Support for the neutral mutation theory. Evolutionary Biology 23, 271-367.

Wilkens, H. 2001. Convergent adaptations to cave life in *Rhamdia laticauda* catfish group (Pimelodidae, Teleostei). Environmental Biology of Fishes 62, 251-261.

Wilkens, H. 2005. Fish, pp. 241-251. In Encyclopedia of Caves (Culver, D.C., & W.B. White, eds). Elsevier Academic Press, London.

Wilkens, H. 2010. Genes, modules and the evolution of cave fish. Heredity 105, 413-422.

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