Toxic hydrogen sulfide and dark caves: life-history adaptations in a livebearing fish (Poecilia mexicana, Poeciliidae)

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Abstract. Life-history traits are very sensitive to extreme environmental conditions, because resources that need to be invested in somatic maintenance cannot be invested in reproduction. Here we examined female life-history traits in the Mexican livebearing fish Poecilia mexicana from a variety of benign surface habitats, a creek with naturally occurring toxic hydrogen sulfide (H2S), a sulfidic cave, and a non-sulfidic cave. Previous studies revealed pronounced genetic and morphological divergence over very small geographic scales in this system despite the absence of physical barriers, suggesting that local adaptation to different combinations of two selection factors, toxicity (H2S) and darkness, is accompanied by very low rates of gene flow. Hence, we investigated life-history divergence between these populations in response to the selective pressures of darkness and/or toxicity. Our main results show that toxicity and darkness both select for (or impose constraints on) the same female trait dynamics: reduced fecundity and increased offspring size. Since reduced fecundity in the sulfur cave population was previously shown to be heritable, we discuss how divergent life-history evolution may promote further ecological divergence: for example, reduced fecundity and increased offspring autonomy are clearly beneficial in extreme environments, but fish with these traits are outcompeted in benign habitats.

Key words: Atlantic molly; cave fish; ecological speciation; extremophile teleosts; livebearing fish; matrotrophy; Poecilia mexicana; reproductive life history; Tabasco, southern Mexico.

INTRODUCTION

Life-history traits in extreme environments

Organisms that live in extreme habitats (e.g., due to toxins/toxicants) usually evolve costly adaptations to cope with the physiochemical stressors imposed by the environment (Townsend et al. 2003). When first encountered, these stressors lower an individual’s fitness and, thus, can have a profound influence on reproductive life-history strategies (Sibly and Calow 1989, Stearns 1992, Roff 2002). Since protecting oneself from a stressful environment by excreting or neutralizing toxins (for a review see Sibly and Calow 1989) or via behavioral adaptations (e.g., Plath et al. 2007b, Tobler et al. 2009b) is energetically costly, these processes decrease the resources and energy that can be invested elsewhere, e.g., into reproduction. Several studies have demonstrated phenotypic responses to toxins that exemplify the costs of dealing with environmental stress (guppies, Poecilia reticulata, Uviovo and Beaty 1979; Onychirus armatus, Collembola, Bengtsson et al. 1985; pond snails, Lymnaea stagnalis, Gérard et al. 2005).

Besides toxins and toxicants, perpetual darkness as in cave ecosystems can represent an extreme condition for typical surface-dwelling organisms (Tobler et al. 2006). Cave animals are widely used model organisms to study the evolutionary effects of permanent darkness (e.g., Culver 1982, Culver et al. 1995, Jeffery 2005), and their life-history traits are characterized by switches toward longer life and generation times, older age at first reproduction, increased brood care behavior, as well as decreased fecundity combined with an increase in individual offspring size (reviews in Parzefall 2000, Culver 2005). These traits are often interpreted as adaptations to continuous food scarcity (Culver 1982, 2005, Parker and Begon 1986, Hüppop 2000), because caves are usually nutrient-poor due to a lack of photoautotrophic primary production and reliance on nutrient influx from epigean habitats (Poulson and Lavoie 2000).

Adaptive trait divergence in extremophile Poecilia mexicana

On a plateau in Tabasco (southern Mexico), two populations of the widespread Atlantic molly (Poecilia mexicana) have colonized subterranean watercourses,
the Cueva del Azufre (Gordon and Rosen 1962) and the Cueva Luna Azufre (Pisarowicz 2005, Tobler et al. 2008c). These two limestone caves are unique, because they are the only known caves inhabited by poeciliids. Moreover the watercourses of the cave plateau are characterized by the presence or absence of another physiochemical stressor: naturally occurring hydrogen sulfide (H$_2$S), and all combinations of surface/cave and non-sulfidic/sulfidic habitat types are found (Gordon and Rosen 1962, Tobler et al. 2008c). Hydrogen sulfide is acutely toxic to metazoans (Grieshaber and Völkel 1998) and leads to extreme hypoxia in the water (Tobler et al. 2006, 2009b). Poecilia mexicana from toxic habitats (both cave and surface) perform aquatic surface respiration (ASR) to exploit the more oxygenated (and thus less sulfidic) topmost layer of the water column (Plath et al. 2007b, Tobler et al. 2009a). Hence, mollies from the cave plateau have to cope with the adverse effects of two strong selective forces: darkness and toxicity (Tobler et al. 2006, 2008a, Plath et al. 2007a).

Previous studies have demonstrated evolutionary responses of P. mexicana to these two environmental factors in morphological, physiological, and behavioral traits (Zeiske 1968, Parzefall 1970, Tobler et al. 2008c). Common-garden rearing shows that divergence in the expression of several morphological traits clearly has a heritable basis, because they are also expressed when fish are reared in light for several generations (Parzefall 1970, 2001, Tobler et al. 2008a; Plath and Tobler 2010). Not surprisingly, ecological diversification even over extremely small spatial scales (sometimes only few hundred meters) is reflected by pronounced genetic differentiation (determined on the basis of nuclear microsatellite markers and mitochondrial DNA sequence divergence; Plath et al. 2007a, Tobler et al. 2008a). Gene flow between adjoining, but ecologically divergent habitat types on the cave plateau is indeed very low despite the absence of physical barriers (Plath et al. 2007a, Tobler et al. 2008a, 2009b).

Low gene flow suggests local adaptation to ecological gradients along two axes, (1) non-sulfidic/sulfidic and (2) light/darkness, and strong selection against dispersing, nonadapted individuals (Nosil et al. 2005, Tobler et al. 2008a, 2009b). For example, laboratory-reared cave mollies from the Cueva del Azufre have a higher H$_2$S resistance than fish from non-sulfidic surface streams (Peters et al. 1973; reanalyzed in Plath and Tobler 2010). Hence, this system provides a good opportunity to study the underlying mechanisms at work during ecological speciation (Rundle and Nosil 2005, Plath et al. 2007a, Tobler et al. 2008a; Tobler and Plath, in press).

**Life-history evolution in extremophile P. mexicana**

Since mechanisms that ensure survival in the face of stress can be demanding of resources, this can select for changes in life histories and cause local adaptation, ultimately leading to profound effects on fitness if dispersing individuals find themselves in a habitat type to which they are not locally adapted. However, except for a preliminary study on fecundity differences between the toxic cave and one of the surrounding nontoxic surface habitats (Riesch et al. 2009a), life histories have not been studied so far in this system. Here we examined female reproductive life-history traits in P. mexicana from four different habitat types that provided us with a natural 2 × 2 factorial design of two environmental stressors (darkness and toxic H$_2$S), in which the same fish species inhabits all possible factor combinations. We asked two related questions: (1) What are the general and site-specific differences between the reproductive life histories of P. mexicana from the four different habitat types? (2) Can we determine which life-history traits are selected for/constrained by toxicity and which by darkness?

**METHODS**

**Study system**

The Atlantic molly, Poecilia mexicana, is a widespread freshwater fish, living along the Atlantic coastal drainages of Mexico, and it exhibits wide habitat tolerances. It can be found in first-order through third-order streams, creeks, brooks, lakes, springs, and coastal lagoons, as well as in fresh to brackish waters (Miller 2005). There is also some evidence for the existence of two subspecies: P. mexicana limantouri, inhabiting northern Mexico, and P. mexicana mexicana, occurring south of central Mexico (e.g., Menzel and Darnell 1973, Turner et al. 1983; but see Brett and Turner 1983); however, support for both is still ambiguous, and these designations are rarely used in the literature. Two populations of Poecilia mexicana (the so-called cave mollies; see Plate 1) have colonized limestone caves near Tapijulapa in Tabasco, southern Mexico: the Cueva del Azufre and the Cueva Luna Azufre (Gordon and Rosen 1962, Tobler et al. 2008c). Inside the Cueva del Azufre, several springs discharge water that is rich in hydrogen sulfide (H$_2$S), so cave mollies in the Cueva del Azufre not only live in permanent darkness (Parzefall 2001), but also have to cope with the adverse effects of a naturally occurring toxicant (Tobler et al. 2006) and thus face two strong selective forces (Plath et al. 2007a, Tobler et al. 2008a). Recently, mollies have also been described from a second, non-sulfidic cave, the Cueva Luna Azufre (Pisarowicz 2005, Tobler et al. 2008c). This cave is located only a few hundred meters from the Cueva del Azufre, but a small valley separates the two caves and there is no gene flow between them (Tobler et al. 2008a). Even though the water inside the Luna Azufre is non-sulfidic, the inhabiting mollies face permanent darkness as a potentially strong selective force (Tobler et al. 2008a).

Both caves drain into the El Azufre, which is a sulfidic surface creek that contains high concentrations of hydrogen sulfide. Several sulfidic and non-sulfidic springs discharge into the El Azufre (also upstream
from the Cueva del Azufre), which meanders through the valley separating both caves and eventually drains into the Río Oxolotán (Tobler et al. 2008a). The Río Oxolotán river system consists of numerous non-sulfidic surface habitats (including the Río Amatan and Arroyo Bonita sites sampled for this study) on and around the cave plateau. Fish communities in the Río Oxolotán and its tributaries are complex, but *P. mexicana* appears to be the only fish capable of coping with the conditions of all habitats, including the three described extreme habitats (Tobler et al. 2006, 2008c). Both caves, the El Azufre, and the Río Oxolotán river system are part of the Río Grijalva/Usumacinta drainage (Miller 2005).

**Study populations**

Individual *P. mexicana* were collected in June 2007 and January 2008 in and around the caves as well as in September 2007 in northeastern Mexico and were field preserved in 10% formaldehyde. Cave mollies were collected in the cave chambers V and X of the Cueva del Azufre (Gordon and Rosen 1962) and in the accessible parts of the Cueva Luna Azufre (Tobler et al. 2008a).

Surface fish were collected from the sulfidic surface stream (El Azufre), and the non-sulfidic Río Amatan and Arroyo Bonita. The Arroyo Bonita is a tributary of the Río Oxolotán that is of similar size and structure as the El Azufre. The Río Amatan, on the other hand, is a typical river. It joins the Río Oxolotán, into which El Azufre also drains, ~2 km downstream from the sample site. (For a map, GPS coordinates, and a distance matrix of these sites refer to Tobler et al. [2008a]). Henceforth, the Arroyo Bonita and Río Amatan sites will be referred to as the southern Mexican (SM) sites.

We included several sites from northeastern Mexico (NM) as independent examples of *P. mexicana* populations from non-sulfidic, normoxic surface systems to examine whether life histories of the extromphile populations from the cave plateau deviate from the usual within-species variation. *Poecilia mexicana* were collected from two different drainages: (1) the Río Soto La Marina drainage (NM1, two sites, Río Purificación at Barretal [24°4′42.85″ N, 99°7′21.76″ W] and Río Purificación at Nuevo Padilla [24°2′35.59″ N, 98°54′15.98″ W]) and (2) the Río Tames/Río Pánuco drainage (NM2, two sites, Río La Bomba [22°3′22.32″ N, 98°9′40.01″ W] and an irrigation ditch west of Ebano along MX70 [22°9′0.74″ N, 98°30′12.36″ W]; Fig. 1). Our northernmost sampling points in the sub-tropics (NM1) were located ~1500 km north of the tropical cave plateau in Tabasco (Fig. 1).

In summary, our sampling scheme included four different habitat types and provided us with a natural 2 × 2 design, where the same fish species inhabits all possible combinations of the two environmental factors light and toxicity: (1) NM1, NM2, and SM (all light/nontoxic), (2) El Azufre (light/toxic), (3) Luna Azufre (dark/nontoxic), and (4) Cueva del Azufre (dark/toxic).

**Life-history analyses**

Following the protocol of Reznick and Endler (1982), all preserved fish were weighed and measured for standard length. The reproductive tissue and, if present, all developing offspring were removed. Offspring were counted and their stage of development determined (Reznick 1981, Reznick and Endler 1982, Haynes 1995, Reznick et al. 2002). Somatic tissues, reproductive tissues, and embryos were then dried for 10 d at 40°C and weighed again. To assess female and embryo condition, somatic tissues and embryos were rinsed six times for at least six hours in petroleum ether to extract soluble nonstructural fats (Heuett et al. 1995, Marsh-Matthews et al. 2005) and were then dried again and reweighed. Furthermore, we calculated reproductive allocation (RA) by dividing offspring mass by the sum of offspring mass plus somatic dry mass (Reznick and Endler 1982).

**Maternal provisioning**

To evaluate the mode of maternal provisioning, the matrotrophy index (MI) was calculated (utilizing the slopes and regression coefficients from the regression analysis described below). The MI equals the estimated dry mass of the embryo at birth divided by the estimated dry mass of the oocyte at fertilization (e.g., Reznick et al. 2007). If the eggs are fully provisioned by yolk prior to fertilization (lecithotrophy), then we would expect the embryos to lose 35–40% of their dry mass during development (MI between 0.60 and 0.65; Scrimshaw 1945, Wourms et al. 1988). On the other hand, in the case of continuous maternal provisioning even after fertilization (matrotrophy), one would expect the embryos to lose less mass (MI between 0.65 and 1.00) or even to gain mass during development (MI ≥ 1.00; e.g., Reznick et al. 2002).

**Statistical analyses**

All statistical analyses were conducted using SPSS 16.0.2 for Mac (SPSS, Chicago, Illinois, USA).

**Discriminant function analyses (DFAs).**—To provide an intuitive metric with respect to the magnitude of life-history divergence, we conducted several discriminant function analyses (DFA). We used a jackknife ("leave-one-out") sampling scheme as a cross-validation technique (i.e., each case is classified by the functions derived from all cases other than that case). For each DFA, a priori probabilities were calculated based on group sizes, and an overall classification success was calculated.

The first DFA tested for the discrimination power of life-history parameters along the two environmental factors toxicity and light condition, as well as along geographic distance. The grouping variables were Cueva del Azufre (dark/toxic), Luna Azufre (dark/nontoxic), El Azufre (light/toxic), SM (light/nontoxic), NM1 (light/nontoxic), and NM2 (light/nontoxic). To accommodate the potential effects of standard length (SL) and embryo stage on life-history traits, we used residuals from a
The preparatory general linear model (GLM) as dependent variables (note that the preparatory GLM used here is different from the analytical GLM described in Appendix A). In this multivariate GLM, SL and embryo stage were included as covariates. The dependent variables for the GLM and their residuals for the DFA were female lean mass (in grams), female fat content (as a percentage), fecundity (number of offspring), reproductive allocation (as a percentage), embryo lean mass (in milligrams), and embryo fat content (as a percentage).

In a second set of DFAs, we tested for classification success based on habitat type within southern Mexican populations alone. The same dependent variables (as residuals) were used as in the previous analysis; however, this time the grouping variables were dark/toxic (Cueva del Azufre), dark/nontoxic (Luna Azufre), light/toxic (El Azufre), and light/nontoxic (SM). Again, the preparatory multivariate GLM included the covariates SL and embryo stage.

For all preparatory multivariate GLMs, all dependent variables and covariates were either log-transformed or arcsine-transformed (in the case of percentages) to control for potential nonlinear relationships between the variables.

Principal components analysis (PCA).—We conducted PCA to test for classification based on life-history characters without defining groups prior to analysis. The dependent variables (female lean mass, female fat content, fecundity, reproductive allocation, embryo lean mass, and embryo fat content) were again the residuals from a preparatory mutivariate GLM with the factor drainage and the covariates SL and embryo stage.

Maternal provisioning (matrotrophy index)

Maternal provisioning was evaluated by analyzing the relationship between embryonic dry mass and stage of development by means of linear regression analysis (e.g., Reznick et al. 2002). ANCOVA was used to test for slope heterogeneity.

RESULTS

Condition.—Female fat content showed more variation between populations from southern Mexico (range of means, 2.74–9.93%) than between benign surface populations across Mexico (3.47–9.93%); however, fish from extreme environments did not exhibit lower fat contents than found among those from benign surface habitats (Table 1). Female lean mass showed the opposite pattern and was generally lower in extreme habitats (southern Mexico, 0.13–0.31 g; benign surface populations, 0.31–0.71 g; Table 1).

Reproduction.—All reproductive traits showed higher variability within southern Mexican populations (SM and extreme habitats) than between populations from benign surface habitats across Mexico (e.g., range of mean fecundity, 5.79–20.14 vs. 9.96–20.14 offspring/female; embryo fat content, 14.12–20.75% vs. 16.37–20.75%; Table 1). The lowest fecundity was found in females from the toxic Cueva del Azufre (5.79 offspring/female, corrected for size), and the highest was found in females from SM (20.14 offspring/female; Table 1).

Fig. 1. Map of the collection sites of the Atlantic molly (Poecilia mexicana) in Mexico. Major cities are in gray. Sample sites are: 1 and 2, Rio Soto la Marina drainage (NM1); 3 and 4, Rio Tamesi/Panuco drainage (NM2); 5, the southern Mexican Rio Grijalva/Usumacinta drainage (SM), including the cave plateau.
Table 1. Descriptive statistics for life-history traits of female Atlantic molly (Poecilia mexicana) from four different habitat types and three different drainages in Mexico.

<table>
<thead>
<tr>
<th>Habitat type and population</th>
<th>SL of reproducing females (mm)</th>
<th>Female fat content (%)</th>
<th>Female lean mass (g)</th>
<th>Fecundity (no. offspring)</th>
<th>RA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark/toxic, Cueva del Azufre</td>
<td>37/83</td>
<td>36.97 ± 4.59</td>
<td>6.21 ± 4.41</td>
<td>0.20 ± 0.07</td>
<td>5.79 ± 7.91</td>
</tr>
<tr>
<td>Dark/nontoxic, Luna Azufre</td>
<td>31/47</td>
<td>31.26 ± 3.57</td>
<td>8.52 ± 4.99</td>
<td>0.13 ± 0.04</td>
<td>12.89 ± 8.80</td>
</tr>
<tr>
<td>Light/toxic, El Azufre</td>
<td>27/71</td>
<td>31.44 ± 4.40</td>
<td>2.74 ± 2.15</td>
<td>0.17 ± 0.09</td>
<td>14.67 ± 8.63</td>
</tr>
<tr>
<td>Light/nontoxic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.29 ± 4.15</td>
</tr>
</tbody>
</table>

SM: southern Mexican Río Grijalva/Usumacinta drainage; NM₁: northern Mexican Río Soto la Marina drainage; NM₂: northern Mexican Río Tames/Panuco drainage.

Notes: For sample sizes, the numerator corresponds to females with developing young (= effective sample size), and the denominator equals the total number of dissected females. Other than estimated embryo dry mass at birth and matrotrophy index, values are expressed as mean ± SD. Values for estimated embryo dry mass at birth are estimated marginal means ± SD from a general linear model with standard length (SL) as a covariate. Reproductive allocation (RA) is the proportion of total dry mass that consists of developing embryos. Estimated embryo dry mass at birth is the estimated mass at stage 6 = neonate after Reznick (1981) using the slope and constant from the regression between embryonic dry mass and stage of development. Matrotrophy index is the estimated dry mass at birth divided by dry mass at fertilization (using the slope and constant from the regression between embryonic dry mass and stage of development). Population abbreviations are: SM, southern Mexican Río Grijalva/Usumacinta drainage; NM₁, northern Mexican Río Soto la Marina drainage; NM₂, northern Mexican Río Tames/Panuco drainage.

Whereas offspring number (fecundity) decreased in the dark habitats, offspring size (embryo lean mass) increased greatly (see estimated embryo dry mass at birth in Table 1). Embryo fat content was lowest in the caves and highest in the surface habitats (Table 1). On the other hand, offspring from sulfidic habitats were heavier than offspring from non-sulfidic habitats (see estimated embryo dry mass at birth in Table 1), with offspring from the sulfidic Cueva del Azufre being twice as large as the largest offspring from benign surface habitats.

**Discriminant function analyses.**—The first DFA (based on extreme habitats and non-extreme surface drainages) classified 75.5% of the females into the correct group (Fig. 2a). The variables with the most discriminatory ability were embryo lean mass (Wilks’ lambda = 0.441, $F_{5,198} = 50.266$, $P < 0.001$) and fecundity (Wilks’ lambda = 0.511, $F_{5,198} = 37.874$, $P < 0.001$). Fig. 2a shows that discriminant function 1 mainly separates cave from surface habitats, while discriminant function 2 partially corresponds to the separation based on geographic distance. Interestingly, the El Azufre population is clearly separated from the closest non-sulfidic surface habitat (SM), but rather grouped together with the two northern Mexican drainages NM₁ and NM₂. This disrupts the otherwise clear separation of northern from southern Mexican sites along discriminant function 2.

The second DFA was based on habitat type within southern Mexican populations alone and classified 82.8% of the females into the correct group (Fig. 2b). The variables with the most discriminatory power were again embryo lean mass (Wilks’ lambda = 0.416, $F_{1,153} = 71.589$, $P < 0.001$) and fecundity (Wilks’ lambda = 0.439, $F_{1,153} = 65.070$, $P < 0.001$). Fig. 2b shows that discriminant function 1 mainly separates cave from surface habitats, while discriminant function 2 corresponds to the separation of toxic vs. nontoxic habitats.

Fig. 2. (a) Discriminant function analysis (DFA) for separation of drainages/extreme habitats based on female Atlantic molly life-history traits while controlling for female size (standard length, SL) and embryo stage. (b) DFA for separation among habitat types based on female life histories while controlling for female size (SL) and embryo stage. Values are group centroids ± SD. See Fig. 1 for an explanation of sample site abbreviations.
As a post hoc analysis, we reran the second DFA twice to test for the independent effects of first, toxicity only (two groups, toxic vs. nontoxic habitats) and second, light condition only (two groups, dark vs. light habitats). Based on toxicity alone, the classification success increased to 86.0% (embryo lean mass, Wilks’ lambda = 0.661, $F_{1, 155} = 79.322$, $P < 0.001$; fecundity, Wilks’ lambda = 0.757, $F_{1, 155} = 49.729$, $P < 0.001$), but classification based on light condition alone was 93.6% successful (fecundity, Wilks’ lambda = 0.557, $F_{1, 155} = 123.101$, $P < 0.001$; embryo lean mass, Wilks’ lambda = 0.619, $F_{1, 155} = 95.526$, $P < 0.001$).

Principal components analysis.—The strong differentiation between cave and surface habitats was clearly reflected in the PCA (principal component 1), whereas differentiation based on toxicity was rather weak (Fig. 3). The first two components of the PCA accounted for 62% of the variance. As in the DFAs and GLMs (see Appendices A and B), the two life-history variables that accounted for most of the variation along principal component 1 were embryo lean mass and fecundity (Fig. 3).

Maternal provisioning

In all six populations of *P. mexicana*, embryo dry mass decreased during embryo development (Fig. 4). The estimated MI was highest in the Luna Azufre (0.85) and lowest in the Cueva del Azufre (0.56) and SM (0.57), whereas all other drainages/extreme habitats ranged between 0.61 and 0.67 (Table 1, Fig. 4). Differences in the decrease of embryo dry mass were reflected in a significant interaction of drainage/extreme habitat by embryo stage (ANCOVA on log-transformed data: $F_{5, 180} = 3.854$, $P = 0.002$).

**DISCUSSION**

**Life-history differentiation in extremophile *P. mexicana***

In southern Mexico, *P. mexicana* has colonized four different habitat types. This provided us with a natural 2 × 2 design in which the same fish species inhabits watercourses with all possible combinations of the two abiotic environmental factors, toxicity (H$_2$S) and light/darkness. Unsurprisingly, we found considerable variation in life-history traits comparing benign surface habitats across Mexico, which are up to 1500 km apart from one another and in separate climate zones (subtropical in northern Mexico vs. tropical in southern Mexico). However, the pronounced variation seen in extremophile *P. mexicana* on a very small geographic scale on the cave plateau (several hundred meters) clearly follows a different selective trajectory. Hence we argue that the observed pattern of strongly divergent life histories in southern Mexican populations is not one of “isolation by distance” (Slatkin 1993), but rather caused by “isolation by adaptation” (Nosil et al. 2008, 2009). Along with previous studies focusing on divergence in other traits (e.g., morphological or behavioral divergence), our current results support the hypothesis that toxicity and darkness are examples of two strong selective forces that shape adaptations for immediate survival (Plath et al. 2007b, Tobler et al. 2008a, 2009b) and successful reproduction. Consequently, our discussion will focus primarily on differences between extremophile and non-extremophile *P. mexicana* from southern Mexico; analysis of general life-history variation in *P. mexicana* along biogeographic regions will be the focus of another study, including more populations across Mexico (R. Riesch, unpublished data).

*Poecilia mexicana* from the four different habitat types (light/nontoxic, light/toxic, dark/nontoxic, and dark/toxic) exhibited vast differences in various life-history traits. Even though a number of female life-history traits showed variation between habitat types, fecundity and offspring size were the traits that diverged most strongly (see also Appendix A: Table A1 for a

**FIG. 3.** Principal component analysis based on life-history traits of female Atlantic mollies. The nontoxic surface habitats were SM, NM$_1$, and NM$_2$ (see Fig. 1 for an explanation of sample site abbreviations). These were contrasted against the toxic cave (Cueva del Azufre), the toxic surface habitat (El Azufre), and the nontoxic cave (Luna Azufre).
multivariate GLM on the various dependent variables considered in this study) and both darkness and toxicity select for (or impose constraints on) the same trait dynamics: reduced fecundity and increased offspring size.

**Reduced resource availability as a selective factor?**

Caves are generally described as being resource-limited, because they lack photoautotroph primary production and thus rely on organic influx from surrounding surface habitats (Hüppop 2000, Poulson and Lavoie 2000). Sulfidic habitats, on the other hand, have been described to be nutrient-rich due to chemolithotroph primary production (Langecker et al. 1996). So how does this relate to the sulfidic Cueva del Azufre? Like other sulfidic habitats, the Cueva del Azufre is also resource-rich; however, this does not necessarily mean that cave mollies have a surplus of resources to put into reproduction or growth. Detoxifying H$_2$S is energetically costly (Sibly and Calow 1989), so that acquired resources are most likely immediately funneled into physiological detoxification of H$_2$S. Additionally, *P. mexicana* in sulfidic habitats rely on aquatic surface respiration (ASR) to exploit the more oxygenated (and thus less sulfidic) topmost layer of the water column (Plath et al. 2007b, Tobler et al. 2009a). In a recent study, we could demonstrate that time budgets of fish from sulfidic habitats are dominated by ASR, such that only a small proportion of their time can be spent feeding (for a more detailed discussion see Tobler 2008, Tobler et al. 2009a). These processes may actually lead to resource limitation in an otherwise resource-rich environment. Therefore, even if fat content did not differ between extreme and nontoxic surface habitats (but see Plath et al. 2005, Tobler et al. 2006, 2008c, Tobler 2008), the lower somatic lean masses found in extreme environments are still best explained by resource limitation.

Moreover, it should be noted that the absence of light in the two caves could make resource acquisition more difficult for cave mollies, regardless of the actual resource level in the respective cave. In fact, preliminary data suggest that cave mollies may not necessarily be more efficient in finding and acquiring food in darkness than surface mollies (R. Riesch, unpublished data).

**Reduced fecundity**

Toxicity and darkness both lead to reduced fecundity, while at the same time offspring size increases. Reduced fecundity and increased offspring size in fish from the Cueva del Azufre clearly have a heritable component since it was found also in fish that were reared under common-garden conditions over several generations (Riesch et al. 2009b, in press b), which strongly suggests

**Fig. 4.** Scatter plots of mean embryo dry mass vs. stage of development of Atlantic mollies for four different habitat types. Stages are determined on a progressive scale with the earliest (5) being the neurula stage and the oldest (50) being equivalent to embryos that are ready to be born (after Haynes 1995, Reznick et al. 2002). Matrotrophy index (MI) is the estimated dry mass at birth divided by dry mass at fertilization.
adaptive divergence as an evolutionary response to strong selection.

Reduced fecundity has been found in most comparisons of epigean and hypogean organisms (Christiansen 1965, Culver et al. 1995, Hüpppop 2000, Parzefall 2000, Culver 2005), such as some cave fishes (e.g., cave amblyopsids [Poulsen 1963]; a characid, Astyanax mexicanus [Hüpppop and Wilkens 1991]; catfish, Trichomycterus chaberti [Pouilly and Miranda 2003]), and this was termed the “equilibrium strategy” (Winemiller 1992). In our system, P. mexicana from cave habitats also exhibit the same life-history patterns with reduced fecundity and larger offspring, and fish with the lowest fecundity and largest offspring size come from the Cueva del Azufre, where both factors (darkness and toxicity) act in concert. However, it is interesting to note that the combination of darkness and toxicity results in lower fecundity (and, at least qualitatively, larger offspring size) than can be explained by a purely additive effect of both environmental stressors (as indicated by a significant interaction effect of light condition by toxicity for fecundity in Appendix A: Table A1).

Selection on increased offspring size and autonomy

Given that fish from the sulfidic surface habitats, as well as from the non-sulfidic and sulfidic caves, are essentially energy-limited (regardless of the actual resource availability of the habitat), how exactly can reduced fecundity and large offspring size be adaptive?

Equilibrium strategists make a large investment into individual offspring to promote juvenile survivorship in crowded, resource-limited, and/or otherwise stressful environments (Winemiller 1992). In a recent paper, Bashey (2008) found supporting evidence for this in guppies, where larger offspring have a competitive advantage in crowded environments. Both the Luna Azufre and the Cueva del Azufre are characterized by areas of extremely high population densities (Tobler et al. 2006). In a recent study, Riesch et al. (2009b) argued that reduced fecundity is most likely caused by selection on offspring size in our study system, which would increase juvenile survivorship, for example, by decreasing the risk of starvation and increasing offspring mobility for more efficient foraging (Poulson and White 1969, Pouilly and Miranda 2003). Furthermore, intracohort cannibalism is widespread among poeciliids (e.g., Gambusia affinis [Dionne 1985, Meffe and Crump 1987]; X. hellerii [Jones et al. 2007]; Poecilia latipinna and P. formosa [Hubbs and Schlupp 2008]). In western mosquitofish (G. affinis), low food availability increased the rate of cannibalism (Dionne 1985), and bigger offspring are less likely to be preyed upon by conspecific adults in topminnows (Poeciliopsis spp. [Weeks and Gaggiotti 1993]) and mosquitofish (Gambusia spp. [Hubbs 1991, 1992]). Even though studies on cannibalism are as yet lacking in this system, it has long been proposed to be a major factor in the Cueva del Azufre, and bigger offspring are most likely less prone to being preyed upon by other adult P. mexicana.

Finally, increased offspring size leads to a decreased body surface:volume ratio, so less surface area per volume of body tissue will be exposed to hydrogen sulfide in toxic environments. Furthermore, larger offspring probably have lower metabolic rates and lower oxygen consumption per volume body tissue,
which would be generally adaptive in resource-limited environments. Hence, larger offspring most likely perform ASR more efficiently.

**General life-history evolution in caves**

In addition to increased offspring size and reduced fecundity, a variety of other life-history traits have been described to characterize cave organisms: reduced reproductive effort, delayed reproduction, reduced growth rates, decreased proportion of the population breeding at any given time, and increased life span, which also results in an increase in the maximum number of broods (Parzefall 2000, Culver 2005). Compared to their closest relatives, *P. mexicana* from southern Mexican nontoxic surface waters (SM), cave mollies from both caves did indeed have a lower proportion of breeding females at any given time (but see Riesch et al. 2009b). On the other hand, if one takes the northern Mexican populations into account (NM1 and NM2), this pattern did not hold.

Furthermore, reproductive allocation (our measure of reproductive effort) also did not quite follow the predicted pattern: while RA was lower in mollies from the nontoxic Luna Azufre, it was actually highest in mollies from the toxic Cueva del Azufre. Toxicity, however, seems to select for increased reproductive allocation in *P. mexicana* (it is interesting to note that this is not the case in two other sulfur-endemic poeciliids, *Gambusia eurystoma* and *Poecilia sulphuraria*, nor in *Gammarus sexradiata*, which sometimes occurs in sulphidic surface waters [Riesch et al., *in press*]). Hence, it appears as if the toxicity in the Cueva del Azufre exerts stronger selection on increased reproductive allocation, ultimately offsetting the reduction of reproductive allocation induced by the absence of light, thus resulting in a higher reproductive allocation in the Cueva del Azufre than in either the nontoxic cave or the nontoxic surface habitats.

Finally, data on life span, lifetime reproductive effort, maximum number of broods, or age at first reproduction in the *P. mexicana* system are as yet limited. However, preliminary analysis from a common-garden rearing experiment seems to indicate that cave mollies from the Cueva del Azufre are indeed characterized by delayed maturity and reduced growth rates (R. Riesch, *unpublished data*). Therefore, if cave mollies also have a longer lifespan than surface mollies, lifetime reproductive effort and maximum brood number could indeed be larger in both cave molly populations (in particular in cave mollies from the Cueva del Azufre, which are already characterized by increased reproductive allocation per clutch).

**The role of predation in the cave molly system**

Traditionally, a variety of differences in poeciliid behavior, morphology, and life history have been attributed to differences in predatory regimes between populations (e.g., Reznick and Endler 1982, Langerhans et al. 2007). As we have demonstrated elsewhere, there are obvious differences in predator regimes between sulfidic and non-sulfidic habitats, as well as between surface and cave habitats: while fish communities (including piscivorous fishes) are usually complex in benign surface habitats, they are highly reduced in sulfidic and cave habitats, with extremophile poeciliids being the only permanent piscine residents (Riesch et al. 2006, 2009a, Tobler et al. 2006, 2008c). However, this does not mean that extremophile poeciliids are safe from predation. On the contrary, the piscivorous bird community is essentially the same for both types of surface habitats, and bird predation actually appears to be higher in sulfidic surface habitats (Riesch et al. 2009a). This is probably due to extremophile poeciliids having to stay close to the water surface to perform ASR (Plath et al. 2007b, Tobler et al. 2009a), which makes them relatively easy prey for piscivorous birds. The only known predators in the Cueva del Azufre, on the other hand, are giant water bugs (*Belostoma* sp.) and several species of large-bodied spiders (Tobler et al. 2007, 2008b, Tobler 2009; Horstkotte et al., *in press*). These cave predators are abundant, and they seem to exert high predation pressures on cave mollies (Tobler et al. 2007). Hence, cave mollies do not live in predator-free environments, as has been suggested for other cave fishes (Romero and Green 2005).

**Maternal provisioning**

With the exception of Luna Azufre females, all populations had a matrotrophy index between 0.57 and 0.72, which according to this method would be interpreted as pure lecithotrophy. The embryo mass loss during development of 28–43% was well within the range of dry mass loss in other oviparous fishes (25–55%; Wourms et al. 1988). Luna Azufre females (MI = 0.86), however, clearly showed some degree of post-fertilization provisioning. This finding highlights the importance of intraspecific variation when interpreting maternal provisioning in poeciliids, and our results suggest that *P. mexicana* is at least capable of employing so-called “incipient matrotrophy” or dual provisioning (Blackburn 1992; Marsh-Matthews et al., *in press*; Riesch et al., *in press*).

**Divergent life histories and their potential role for reproductive isolation**

We have shown that fish from extreme habitats have lower fecundity and larger offspring than fish from nontoxic surface habitats. Nonetheless, even among extreme habitats fecundity and offspring size vary greatly, with the extreme values being found in the Cueva del Azufre, where both environmental stressors (darkness and toxicity) work in unison. These patterns could be explained by two mechanisms that are not mutually exclusive. It could be possible that some of the observed life-history differences between (in particular) the “seasonless” caves (e.g., Poulson and White 1969,
Culver 2005, Tobler et al. 2006, 2008a) and the surface habitats are simply due to seasonal plastic variation in the latter (e.g., lack of seasonal cues for reproduction in the caves or plastic responses to seasonal fluctuations in the surface habitats; Chapman et al. 1991, Chapman and Chapman 1993, Culver 2005, Tobler et al. 2006, 2008a). The same holds true for the comparison of surface habitats across Mexico. In southern Mexico, we tried to counteract this by sampling during both the dry season (June) and the rainy season (January) in all habitats (both caves, the El Azufre, and SM), but some of the observed variation may still be accounted for by plastic responses to seasonal differences.

However, with regard to fecundity and offspring size we have evidence that plasticity is only part of the explanation: in recent studies we found that reduced fecundity and increased offspring size in fish from the Cueva del Azufre are clearly heritable (Riesch et al. 2009b; Riesch et al., in press). Studies on cave Amblyopsidae have also demonstrated that cave fish life histories are not simply constraints imposed by the environment, but are, at the very least, combinations of environmentally induced constraints and naturally selected evolutionary adaptations (review in Culver 2005). Hence, our results hint at adaptive trait divergence as another possible mechanism of ecological diversification, in which the extent of reduction in fecundity and increase in offspring size is beneficial in the respective extreme habitat, but females will most likely be outcompeted in the “wrong” habitat type. For example, dispersing individuals with low fecundity will be outcompeted in non-extreme habitats, in which females produce far more offspring. Competition by better locally adapted individuals (competitive exclusion) along with increased juvenile mortality (due to the “wrong” offspring size in the “wrong” habitat) most likely results in strongly decreased fitness of dispersing individuals in this system. This, however, has to be put into perspective: when P. mexicana initially invaded the novel extreme environments, ecological divergence most likely caused the original divergence in life-history evolution. Nonetheless, we propose that divergent life histories at this point in time in our study system act as an additional mechanism that, along with trophic (Tobler 2008), morphological (Tobler et al. 2008a, c), and behavioral divergence (e.g., Plath et al. 2005, 2007b), effectively restricts gene flow through direct selection against “migrants” (Plath et al. 2007a, Tobler et al. 2008a, 2009a). In summary, disruptive life-history trait evolution due to local adaptations to different habitat types provides another mechanistic link promoting ecological diversification and, ultimately, parasitic speciation in this system.

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LITERATURE CITED


APPENDIX A

General linear models (GLMs) on female life-history traits and additional matrotrophy analyses (Ecological Archives E091-103-A1).

APPENDIX B

Supplementary information on the discriminant function analyses (DFAs) on female life-history traits (Ecological Archives E091-103-A2).