Toxic hydrogen sulphide and dark caves: pronounced male life-history divergence among locally adapted *Poecilia mexicana* (Poeciliidae)

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Abstract

Chronic environmental stress is known to induce evolutionary change. Here, we assessed male life-history trait divergence in the neotropical fish *Poecilia mexicana* from a system that has been described to undergo incipient ecological speciation in adjacent, but reproductively isolated toxic/nontoxic and surface/cave habitats. Examining both field-caught and common garden–reared specimens, we investigated the extent of differentiation and plasticity of life-history strategies employed by male *P. mexicana*. We found strong site-specific life-history divergence in traits such as fat content, standard length and gonadosomatic index. The majority of site-specific life-history differences were also expressed under common garden–rearing conditions. We propose that apparent conservatism of male life histories is the result of other (genetically based) changes in physiology and behaviour between populations. Together with the results from previous studies, this is strong evidence for local adaptation as a result of ecologically based divergent selection.

Introduction

Life in extreme habitats

Habitats can be considered extreme if certain characteristics of the environment are outside of the range normally experienced by a species and if an organism colonizing this habitat type experiences a reduction in fitness (Calow, 1989; Rothschild & Mancinelli, 2001). Extreme environments vary in the parameters that make them extreme: some can be extremely hot, cold, dry or saline, others are characterized by extreme acidity, radiation or high pressures. Organisms living in these environments are called extremophiles, and even though most extremophiles are microbes (e.g. Rothschild & Mancinelli, 2001; Thomas & Dieckmann, 2002), various extremophile metazoans have been discovered. Among these are icefishes in Antarctic waters, and tubeworms

and crabs at deep-sea hydrothermal vents (e.g. Cavanaugh *et al.*, 1981; Vetter *et al.*, 1987; Kock, 2005a,b; Tobler *et al.*, 2006).

Many extreme environments are characterized by exceptionally high concentrations of hydrogen sulphide (H_2S): deep-sea hydrothermal vents, hydrocarbon seeps, as well as intertidal zones, salt marshes, mudflats and sewage outfalls, where hydrogen sulphide is usually of biogenic origin (Bagarinao, 1992; Grieshaber & Völkel, 1998; McMullin *et al.*, 2000; Theissen & Martin, 2008). Hydrogen sulphide inhibits aerobic respiration owing to its interference with mitochondrial respiration and blood oxygen transport, but also leads to extreme hypoxia in the water (Bagarinao, 1992; Grieshaber & Völkel, 1998). This makes hydrogen sulphide acutely toxic to most metazoans, even in micromolar amounts, and accordingly, pulses of hydrogen sulphide discharge have been reported to be the source of mass mortalities (Grieshaber & Völkel, 1998). An environmental toxicant like hydrogen sulphide that requires energetically costly behavioural (i.e. actively avoiding microhabitats with high levels of toxicity) and physiological adaptations (various forms of detoxification) by animals exposed to it (Tobler *et al.*,

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2009a; Riesch *et al.*, submitted) will have a profound influence on life-history traits in populations experiencing this stressor. If coping with toxicants requires costly adaptations (e.g. Calow, 1989; Sibly & Calow, 1989), then fewer resources are available for reproduction and growth, because more energy is channelled into somatic maintenance (Gray, 1989). Accordingly, organisms exposed to environmental stressors are usually characterized by reduced mean size relative to individuals of the same species from undisturbed habitats (for review, see Gray, 1989). In oviparous fishes, hydrogen sulphide has been reported to cause reduced egg production, lower survival of eggs, reduced size and higher incidence of deformities in hatchlings and reduced gonadosomatic index, but also reduced survival and growth in adult fish. Finally, fish fry are usually several times more sensitive to hydrogen sulphide than juveniles or adults (Bagarinao, 1992).

Ecological speciation in extremophile mollies

All the adverse effects of hydrogen sulphide notwithstanding several species of livebearing fishes (Poeciliidae) have been documented to thrive (and speciate) in highly sulphidic waters. Among them are sulphur endemics like *Gambusia eurystoma*, *Limia sulphurophila* and *Poecilia sulphuraria* (e.g. Tobler *et al.*, 2008c; Plath *et al.*, 2010a; Riesch *et al.*, 2010a), as well as species that are currently undergoing ecological speciation (i.e. in which reproductive isolation is the product of ecologically based divergent selection; Schluter, 2001; Rundle & Nosil, 2005), like certain populations of *Poecilia mexicana* (Plath *et al.*, 2007a, 2010b; Tobler *et al.*, 2008a, 2009b; Riesch *et al.*, 2010b). Of particular interest are *P. mexicana* populations in the Cueva del Azufre system (Tabasco, Mexico), because it is characterized by the simultaneous action of two strong selective forces: permanent darkness and hydrogen sulphide (Gordon & Rosen, 1962; Tobler *et al.*, 2006, 2008d). Within a small geographical scale of only few kilometres, reproductively isolated populations of *P. mexicana* inhabit environments characterized by all possible combinations of these two factors: a toxic cave (Cueva del Azufre, CdA), a nontoxic cave (Cueva Luna Azufre, CLA), a toxic surface stream (El Azufre, EA) and a variety of nontoxic surface habitats in the Río Grijalva/Usumacinta (RGU) drainage (Gordon & Rosen, 1962; Pisarowicz, 2005; Tobler *et al.*, 2006, 2008d). Toxicity (i.e. H_2S) in this system is of volcanic origin (Rosales–Lagarde *et al.*, 2006, 2008) and reaches concentrations of >150 μM in EA and >300 μM in CdA (Tobler *et al.*, 2006, 2008a). All extreme habitats are interconnected, and no physical barriers would prevent individual dispersal and thus gene flow between these populations; however, a small cascade separates all extreme habitats from the RGU sites (for discussion, see Plath *et al.*, 2010b). Strong reproductive isolation among populations from ecologically divergent habitat types

appears to be the result of a combination of natural selection (i.e. direct effects of toxicity and predation that is biased against maladapted individuals) and sexual selection (Tobler, 2009; Tobler *et al.*, 2009b; Riesch *et al.*, 2010d).

Extremophile *P. mexicana* are characterized by site-specific local adaptations in behavioural (e.g. Plath *et al.*, 2004b, 2007c; Plath, 2008; Riesch *et al.*, 2009a; Tobler *et al.*, 2009a), dietary (Tobler, 2008), female life history (Riesch *et al.*, 2009b, 2010a–c), morphological (Tobler *et al.*, 2008a,d) and physiological traits (Plath & Tobler, 2010). Life-history evolution of extremophile female poeciliids is dominated by shifts towards large offspring size and reduced fecundity (Riesch *et al.*, 2009b, 2010a–c), and population differences in those traits have been found to be largely heritable in cave mollies from CdA (Riesch *et al.*, 2009b, 2010c). In fact, relative embryo size in four different poeciliid species can be predicted by hydrogen sulphide concentrations of their respective habitat of origin (Riesch *et al.*, 2010a). However, whether life-history traits other than embryo size/fecundity have a genetic basis is not known, and male life histories in extremophile poeciliids so far have received almost no attention. The present study, therefore, represents the first in-depth analysis of male life-history divergence in these systems. If the offspring size/fecundity trade-off in females is indeed the main target of selection on life-history traits (Riesch *et al.*, 2009b, 2010a–c), then one would predict to find male life-history divergence to be less pronounced in extremophile *P. mexicana*. However, whether other life-history traits are under site-specific selection in males remains to be tested.

Here, we investigated life-history strategies of field-caught and laboratory-reared male *P. mexicana* that originated from four different habitat types: a toxic cave, a nontoxic cave, a toxic surface creek and three nontoxic surface drainages. Specifically, we asked three questions: (i) What are the general and site-specific life-history differences of male *P. mexicana* from the four different habitat types? (ii) Are there certain life-history traits that correspond to the presence or absence of darkness and toxicity? In other words, could darkness and toxicity act on other traits besides the offspring size/fecundity trade-off in females? (iii) Do the observed life-history shifts constitute genetically based divergence or primarily phenotypic plasticity?

Methods

Study system and study populations

Individual *P. mexicana* were collected in June 2007 and January 2008 in the Cueva del Azufre (CdA) system (i.e. in and around the caves) and field preserved in 10% formaldehyde. Because of structural differences between habitats, different sampling methods were employed. In the caves, where the water is very shallow and low

ceilings preclude seining, fish were caught with dip nets (13 × 14 cm, 1-mm mesh-width). In the other habitats, fish were caught using a seine (4 m long, 4-mm mesh-width). Cave mollies were collected in the cave chambers V and X of the CdA (Gordon & Rosen, 1962) and in the accessible parts of the Cueva Luna Azufre (CLA; Tobler *et al.*, 2008a). To increase sample sizes, 13 additional CLA males were collected in September 2008. Surface fish were collected from the sulphidic El Azufre creek (EA), and the nonsulphidic Río Amatan (RA) and Arroyo Bonita (AB). AB is of similar size and structure as EA. RA, on the other hand, is a river. It joins Río Oxolotán (RO), into which EA also drains, approximately 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, AB, RA and RO will be referred to as the Río Grijalva/Usumacinta sites (RGU).

As independent samples of *P. mexicana* populations from nonsulphidic, normoxic surface systems, we further included two different drainages from north-eastern Mexico: the Río Soto la Marina drainage (RSlM; two river sites, for details, see Riesch *et al.*, 2010b) and the Río Carrizal drainage (RC; an irrigation ditch between Nuevo Progresso and Aldama; 23°3′0.00″N, 98°1′56.75″W). Our northernmost sampling points in the subtropics (RSlM and RC) were located approximately 1200–1500 km north of our study sites in tropical Tabasco. This provided us with an opportunity to examine with relative confidence if life histories of the extremophile populations from the cave plateau deviate from the usual within-species variation. Replication of extreme habitats, however, was not possible because the two caves (CLA and CdA) are the only caves known to be inhabited by livebearing fishes, and *P. mexicana* is only known to inhabit sulphidic waters in the El Azufre.

To examine whether life-history divergence between populations is because of phenotypic plasticity or the result of genetic differences, we also evaluated fish from a population-level common garden–rearing experiment (Tobler *et al.*, 2008a). Laboratory stocks of fish were available from all four populations from southern Mexico: the sulphidic cave (CdA), the nonsulphidic cave (CLA), the sulphidic surface (EA) and a nonsulphidic surface habitat (RO). All stocks were founded by dozens of individuals each in January 2006 and maintained as randomly outbred populations in 1000-L flow-through tanks in a temperature-controlled greenhouse at the Aquatic Research Facility of the University of Oklahoma (one tank per population). All stocks were exposed to identical environmental conditions (i.e. natural light cycle, and no H_2S or predators). Algae, detritus and invertebrates were present in the stock tanks, and the diet was supplemented with commercial flake food twice a week. Random samples of males from these stocks were collected in March 2010. At this point, the stocks were established in the laboratory for multiple generations. As for the wild-caught fish, specimens were euthanized using MS222 and fixed in a 10% formaldehyde solution.

In summary, our sampling scheme included field-caught and laboratory-reared fish from four different habitat types and provided us with a natural 2 × 2 factorial design, where the same fish species inhabits habitats with all possible combinations of the two environmental factors 'light' and 'toxicity': (i) light/nontoxic (RGU, RSlM and RC), (ii) light/toxic (EA), (iii) dark/nontoxic (CLA) and (iv) dark/toxic (CdA).

Life-history analyses

Following the protocol of Reznick & Endler (1982), all preserved fish were weighed and measured for standard length. Males were classified as mature based on the morphology of their modified anal fin (i.e. gonopodium). The anal fin in poeciliids undergoes a complex metamorphosis as fish attain maturity, and the endpoint of this metamorphosis provides a reliable index of sexual maturity, allowing us to determine the state of complete maturation based on external cues alone (e.g. Kallman & Schreibman, 1973; Schreibman & Kallman, 1977; Reznick *et al.*, 1993, 2007; Arias & Reznick, 2000). Even though there are slight differences among species (R. Riesch, personal observation), the general metamorphosis is similar to that described by Turner (1941) for *Gambusia affinis*. We also consulted the illustrations of the fully developed gonopodium of several *Poecilia* spp. presented by Rosen & Bailey (1963) to define the endpoint of anal fin metamorphosis for *P. mexicana*. We removed the testis from each male and somatic tissues and testes were then dried for 10 days at 40 °C after which time they were re-weighed. To assess male condition, somatic tissues and testes were rinsed six times for at least six hours in petroleum ether to extract soluble nonstructural fats (Heulett *et al.*, 1995; Marsh–Matthews *et al.*, 2005) and were then re-dried and re-weighed. We thus collected the following male life-history traits: 'male standard length, SL [mm]', 'male dry weight [g]', 'male lean weight [g]', 'male testis dry weight [mg]', and 'male fat content [%]'. Furthermore, we calculated the 'gonadosomatic index (GSI)' by dividing a male's reproductive tissue dry weight by the total body dry weight.

Statistical analyses

All life-history variables were first log-transformed or arcsine-square root-transformed (in the case of percentages) to control for potential nonlinear relationships between the variables, and subsequently z-transformed for standardization (Gotelli & Ellison, 2004). To facilitate all subsequent analyses, we first removed the effects of male body size (SL) on other male traits by means of a preparatory MANCOVA on transformed life-history variables. For this purpose, 'SL' was used as a covariate, and

'testis dry weight', 'male lean weight', 'GSI' and 'male fat content' were the dependent variables. The resulting residuals, along with 'transformed SL', were used as dependent variables in all subsequent analyses.

We first tested male size distributions for normality by means of a Kolmogorov–Smirnov test. Because of limited sample size, we excluded RC from this analysis. In the second step, we compared male body size between habitat types by using a Kruskal–Wallis ANOVA.

General linear models

We tested for differences in male life histories between habitats by means of two multivariate GLMs (MANOVAS). In a preliminary step, data of field-caught and laboratory-reared fish were subjected to two principal component analyses based on a covariance matrix to reduce the data to true dimensionality. Principal axes with an eigenvalue over 1 (two axes each accounting for 64.2% of the total variation in field-caught and 62.8% in laboratory-reared specimen) were retained as life-history variables. In the first model, we tested for differences between field-caught fish, and in the second model, we tested for differences between laboratory-reared fish. For both GLMs, the independent variables were 'light condition (light vs. darkness)' and 'toxicity (nontoxic vs. toxic)'.

Discriminant function analyses

To provide an intuitive metric with respect to the magnitude of life-history divergence, we conducted several DFAs. As a cross-validation technique, we employed a jack-knife (leave-one-out) sampling scheme (i.e. each case was classified by the functions derived from all cases other than the case itself), and *a priori* probabilities were calculated based on group sizes.

In total, we conducted four discriminant function analyses (DFAs). First, we tested for differentiation based on habitat type/drainage to evaluate whether life histories in the extreme habitats fall within the normal between-population variability. For this DFA, all sample sites were included. Second, in two DFAs based only on southern Mexican sites, we then evaluated separation by habitat type based on field-caught fish and separation by habitat type based on laboratory-reared fish. Finally, we tested for a genetic basis to life-history divergence within each habitat type by conducting cross-validation DFA as follows: all data from laboratory-reared fish were withheld, and a DFA model was built on data from field-caught fish only (training data set). The data from the withheld laboratory samples (testing set) were then inserted into the discriminant functions and assigned to the most parsimonious training data set category (Hair *et al.*, 1995).

All statistical analyses were conducted using PASW Statistics 18.0.2 for Mac (SPSS Inc., an IBM company).

Results

Site-specific life histories

Field-caught specimens

Mean standard length for mature males ranged from 21.89 to 42.71 mm and differed significantly between populations (Kruskal–Wallis test: $H_5 = 69.57$, $P < 0.001$), with the smallest males being found in CLA and the largest males coming from RC (Table 1). Male size distribution differed from a normal distribution in the two caves, where size distributions were skewed towards smaller male body sizes, but was normally distributed in surface populations (Kolmogorov–Smirnov test: CdA, $D_{32} = 0.23$, $P < 0.001$; CLA, $D_{18} = 0.21$, $P = 0.04$; EA, $D_{16} = 0.13$, $P = 0.20$; RGU, $D_{32} = 0.10$, $P = 0.20$; RSlM, $D_{13} = 0.14$, $P = 0.20$; Fig. 1).

Males from extreme habitats (CdA, CLA and EA) were characterized by strongly reduced fat content, but lean

Table 1 Descriptive statistics (mean ± SD) for life-history traits of wild-caught and laboratory-reared (parentheses) male *Poecilia mexicana* from four different habitat types and three drainages. CdA, Cueva del Azufre; CLA, Cueva Luna Azufre; EA, El Azufre; RGU, Río Grijalva/Usumacinta drainage; RSlM, Río Soto la Marina drainage; RC, Río Carrizal drainage; GSI, gonadosomatic index.

| | Dark/toxic CdA | Dark/nontoxic CLA | Light/toxic EA | Light/nontoxic | | |
				RGU	RSlM	RC
N	32 (10)	18 (8)	16 (9)	32 (10)	13	7
SL [mm]	30.44 ± 4.09	21.89 ± 2.93	25.94 ± 2.27	28.84 ± 5.58	37.92 ± 4.44	42.71 ± 6.10
	(30.90 ± 2.03)	(28.38 ± 4.63)	(28.22 ± 3.23)	(27.70 ± 6.33)		
Lean Weight* [g]	0.09 ± 0.03	0.16 ± 0.04	0.14 ± 0.04	0.13 ± 0.03	0.17 ± 0.04	0.23 ± 0.04
	(0.08 ± 0.03)	(0.13 ± 0.03)	(0.12 ± 0.03)	(0.13 ± 0.03)		
Fat Content* [%]	4.20 ± 0.03	3.76 ± 0.04	3.22 ± 0.04	9.51 ± 0.03	6.59 ± 0.04	6.49 ± 0.04
	(0.21 ± 0.03)	(1.19 ± 0.03)	(2.92 ± 0.04)	(7.18 ± 0.03)		
Testis Dry	0.71 ± 0.50	1.23 ± 0.59	1.31 ± 0.52	1.30 ± 0.50	0.87 ± 0.58	0.14 ± 0.60
Weight* [mg]	(0.93 ± 0.50)	(1.04 ± 0.50)	(1.59 ± 0.50)	(1.31 ± 0.50)		
GSI*† [%]	0.75 ± 0.57	0.49 ± 0.42	0.95 ± 0.40	0.93 ± 0.57	0.73 ± 0.36	0.60 ± 0.26
	(1.12 ± 0.32)	(0.73 ± 0.28)	(1.42 ± 0.30)	(1.33 ± 0.32)		

*Size-corrected data from a MANCOVA with SL as a covariate (SL = 29.45 mm).
†Gonadosomatic index: proportion of total dry weight which consists of reproductive tissue (testis).
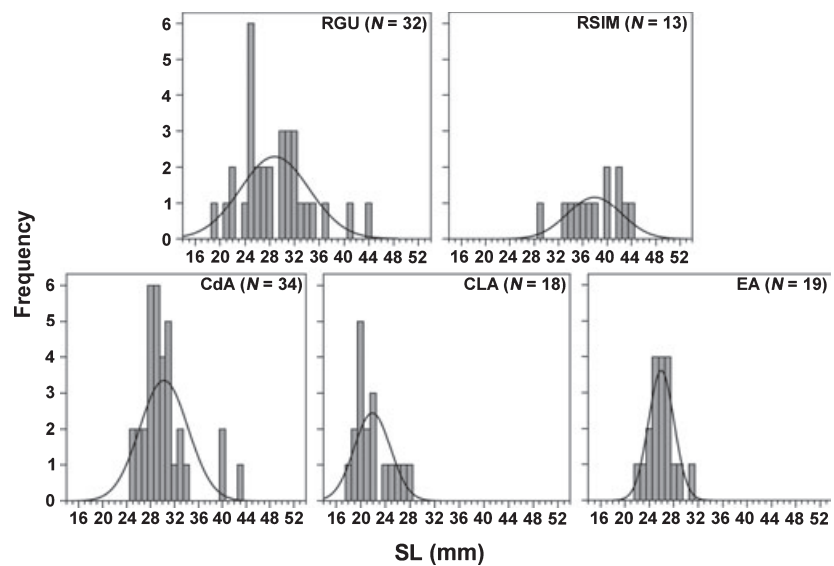
Fig. 1 Size distribution of males ['mature' and 'almost mature' as defined by the morphology of the gonopodium (after Turner, 1941; Rosen & Bailey, 1963)] for the four different habitat types: light/nontoxic (RGU and RSlM), dark/toxic (CdA), dark/nontoxic (CLA) and light/toxic (EA). For visual clarification, a potential normal distribution is superimposed on each graph.

weight, testis dry weight and GSI did not exhibit a predictable pattern (Table 1). Relative to benign surface habitats (RGU, RSlM and RC), however, only males from the toxic cave (CdA) were characterized by consistently divergent life-history shifts. The two ''singular-extreme'' habitats (CLA and EA), on the other hand, differed from nonsulphidic surface habitats in some life-history traits, but were similar to them in others (Table 1).

Laboratory-reared specimens
Mean standard lengths converged in the laboratory (range: 27.70–30.90 mm), and the four populations did not differ significantly from one another (Kruskal–Wallis test: $H_3 = 4.08$, $P = 0.26$). In contrast to the field, the smallest laboratory-reared males originated from RGU, but the largest males were again found in CdA (Table 1), and size distributions did not differ from normal distributions (Kolmogorov–Smirnov test: CdA, $D_{10} = 0.23$, $P = 0.15$; CLA, $D_8 = 0.13$, $P = 0.20$; EA, $D_9 = 0.20$, $P = 0.20$; RGU, $D_{10} = 0.22$, $P = 0.18$).

Overall, convergence of life histories to a common, intermediate phenotype – as a response to common rearing conditions – was not detected (Table 1). Indeed, with a few exceptions (e.g. GSI in CdA or testis dry weight in EA), life-history patterns of laboratory-reared males mirrored the patterns found in wild populations (Table 1). For example, males from all three extreme habitats (CdA, CLA and EA) differed from RGU by storing less-soluble body fat.

Population comparisons

General linear models
In the GLM on field-caught *P. mexicana*, both factors (light condition and toxicity) had a significant influence on male life histories (Table 2a). This indicates that

Table 2 MANOVA results for differentiation of life-history traits as a result of extreme selective forces in field-caught and laboratory-reared male *P. mexicana*. *F*-ratios were approximated using Wilks' λ values. Partial variance was estimated using Wilks' partial η^2.

Factor	F	d.f.	P	Partial variance explained (%)
(a) Field-caught				
Light condition	31.611	2, 113	<0.001	35.9
Toxicity	13.914	2, 113	<0.001	19.8
Light condition × Toxicity	1.843	2, 113	0.163	3.2
(b) Laboratory-reared				
Light condition	13.662	2, 32	<0.001	46.1
Toxicity	4.491	2, 32	0.019	21.9
Light condition × Toxicity	11.312	2, 32	<0.001	41.4

both environmental stressors led to life-history trait divergence.

Life histories of laboratory-reared *P. mexicana* males were affected by both factors (light condition and toxicity) under which the different ecotypes evolved, as well as the interaction of 'light condition by toxicity' (Table 2b). This is evidence that life-history shifts as a response to darkness and/or toxicity were not independent from one another (Table 1).

Discriminant function analyses
The DFA on field-caught fish from across Mexico was able to successfully classify 74.6% of all males into the correct group (compared to the proportional by-chance-accuracy-rate of 20.4%). The variable with the most explanatory power was male standard length (*Wilks' lambda* = 0.38, $F_{5,112} = 36.25$, $P < 0.001$), and CdA and RGU males were the most distinct (84.4% and 81.3% classification success, respectively; Fig. 2). Visual inspection of the discrimination plot demonstrated that
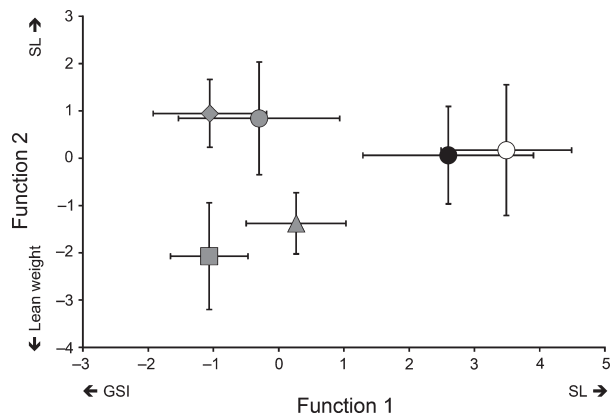
Fig. 2 Group centroids ± SD of discriminant function analyses for separation of populations based on male life-history traits while controlling for the effects of male standard length. Grey circle, light/nontoxic (RGU); black circle, light/nontoxic (RSlM); white circle, light/nontoxic (RC); grey triangle, light/toxic (EA); grey square, dark/nontoxic (CLA); grey diamond, dark/toxic (CdA).

life-history divergence between surface drainages clearly follows a different trajectory than divergence between extreme and benign habitats (Fig. 2).

The DFA on field-caught fish from southern Mexico successfully classified 81.46% of the males into the correct group (compared to the proportional by-chance-accuracy-rate of 27.4%). The variable with the most discriminatory power was male standard length (*Wilks' lambda* = 0.59, $F_{3,94}$ = 21.58, $P < 0.001$; Fig. 2a). Highest classification success was scored for CdA and RGU males (both 84.4%), whereas CLA males scored lowest (72.2%). However, there was no clear effect of darkness or toxicity (Fig. 3a).

The DFA investigating life-history divergence between habitats based on laboratory-reared fish classified 70.3% of the males into the correct group (compared to the proportional by-chance-accuracy-rate of 25.2%). The variable with the most discriminatory power was male fat content (*Wilks' lambda* = 0.47, $F_{3,33}$ = 12.47, $P < 0.001$). CdA males were the most distinct, with 90.0% of individuals being correctly classified, whereas lowest classification success was found for EA males (55.6%). Again, no clear pattern of toxicity and/or darkness emerged, even though darkness appeared to be more important for differentiation than toxicity (Fig. 3b).

Finally, the cross-validation DFA using both data sets was 64.9% successful (compared to the proportional by-chance-accuracy-rate of 27.4%). All CdA males (100%) and most RGU males (90.5) were correctly assigned, whereas assignment of CLA (50.0%) and EA males (11.1%) was weak (Fig. 3c).

Discussion

Comparing benign surface habitats across Mexico, we found variation in male life-history traits between sites
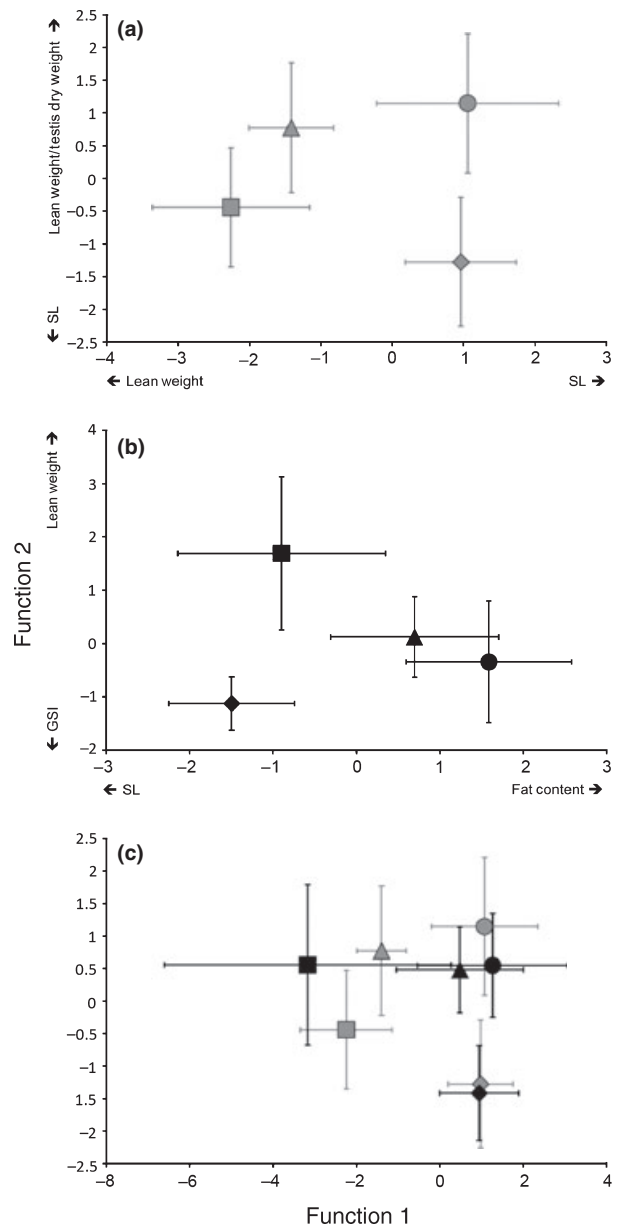

Fig. 3 Group centroids ± SD of discriminant function analyses (DFA) for separation by habitat type based on male life-history traits while controlling for the effects of male standard length. (a) field-caught specimens and (b) laboratory-reared specimens. (c) cross-validation DFA, with data from laboratory-reared males being used to test discrimination based on life-history traits of field-caught males. Circle, light/nontoxic (RGU); triangle, light/toxic (EA); square, dark/nontoxic (CLA); diamond, dark/toxic (CdA); grey, field-caught; black, laboratory-reared.

that are up to 1500 km apart from one another and located in separate climate zones (subtropical northern Mexico vs. tropical southern Mexico). More importantly, we also found pronounced variation on a very small geographical scale in the cave molly system, where sites

are only few 100 metres apart. Similar to females from the same populations (Riesch *et al.*, 2009b, 2010b,c), life-history shifts in extreme habitats clearly follow a different trajectory than life-history variation among benign surface habitats. For our discussion, we will therefore focus entirely on the southern Mexican sites, and general life-history differences of *P. mexicana* from benign surface habitats across Mexico will be the focus of a separate study including more sites throughout Mexico (R. Riesch, unpubl. data).

Although we predicted convergence of male life histories to an intermediate phenotype in the laboratory, most population-specific male life histories were surprisingly conservative and only male size showed signs of convergence. This reveals that most of the male life-history divergence between southern Mexican extreme and benign habitats has a genetic basis. The resolution of male life-history evolution in direct response to toxicity and darkness, however, remains somewhat unclear and unpredictable. This is in clear contrast to female life histories, which showed predictable responses to toxicity and darkness along the offspring size/fecundity trade-off (Riesch *et al.*, 2010a–c).

Site-specific life histories and the effects of darkness and toxicity

Male size in natural populations varied without a predictable pattern. Males of many poeciliid species are characterized by a bimodal size distribution in natural populations (e.g. Kallman, 1989; Reznick *et al.*, 1993, 2007; Kolluru & Reznick, 1996), and it has been shown in *Xiphophorus* spp. that this size polymorphism is controlled by the sex-linked pituitary (P-)locus (Kallman, 1989). This particular pattern of inheritance, however, may not apply to all poeciliids (e.g. Kolluru & Reznick, 1996). For *P. mexicana*, we could not find any evidence for bimodal size distribution in the populations examined in this study. Rather, surface populations were characterized by normal distribution patterns; where distribution patterns deviated from such a normal distribution, this was because of a skew towards smaller males, not bimodal distribution (both caves; Fig. 1).

The main drivers for size distributions of mature males and females in natural populations are sexual selection (e.g. via female mate choice; Ryan *et al.*, 1992) and natural selection (e.g. via predation or resource availability), with the latter often selecting against large, conspicuously ornamented males (e.g. Jennings & Houseweart, 1989; Trexler *et al.*, 1994; Acharya, 1995; Quinn & Kinnison, 1999; Rosenthal *et al.*, 2001). Site-specific combinations of natural and sexual selection are most likely also responsible for the observed patterns in body size of male *P. mexicana* in extreme habitats. Unfortunately, predation has so far not been investigated in the CLA, whereas predation regimes in the CdA and EA are much better understood: even though piscivorous

fishes are absent from all extreme habitats (Tobler *et al.*, 2006; Riesch *et al.*, 2009a), cave mollies in CdA are under strong size- and sex-specific predation through giant water bugs (*Belostoma* sp.; Tobler *et al.*, 2008b; M. Plath & R. Riesch, unpubl. data) and also suffer predation by several arachnid species (Horstkotte *et al.*, 2010), whereas males in EA have to cope with bird predation rates that are up to 20 times higher than in RGU habitats (Riesch *et al.*, 2010d). Herons and egrets have previously been demonstrated to prefer large over small poeciliids as prey (Britton & Moser, 1982; Trexler *et al.*, 1994).

Furthermore, from the perspective of *P. mexicana*, all extreme habitats (EA, CLA and CdA) are energy-limited (Plath & Tobler, 2010), which is known to select for reduced growth rates and smaller size at maturity (e.g. Arendt & Reznick, 2005). Also, a visual preference for large male body size is common in all *P. mexicana* populations (including CdA, CLA and EA), and females from CdA express this preference even under naturally dark conditions ('sensory shift': Plath *et al.*, 2004b, 2007b). CLA females, however, do not express a preference for large males in darkness. Tobler *et al.* (2008e), therefore, argued that relaxed sexual selection may be an additional reason for the general lack of large-bodied males in the CLA population.

In addition to its effects on body size, energy limitation is most likely also an explanation for the low body fat found in males (and females; see Riesch *et al.*, 2010b) from extreme habitats. With regard to energy availability, CLA is a typical cave habitat that lacks photoautotrophic primary production (Hüppop, 2000; Tobler, 2008). For the sulphidic habitats (EA and the CdA), the picture is somewhat more complex, because sulphidic systems are usually characterized by high levels of bacterial chemo-autotrophic primary production (Langecker *et al.*, 1996). However, it is unlikely that *P. mexicana* can fully exploit this energy, because they spend up to 80% of their time performing aquatic surface respiration (ASR; compared to 0% ASR and ca. 60% feeding in nonsulphidic surface habitats; Tobler *et al.*, 2009a). Furthermore, physiological mechanisms for coping with toxicity are usually energetically costly (e.g. Calow, 1989; Sibly & Calow, 1989; Bagarinao, 1992), so *P. mexicana* probably have to channel a large proportion of the total acquired energy into hydrogen sulphide detoxification mechanisms, leaving almost no surplus nutrients for storage as fat reserves (Gray, 1989).

We also discovered reduced GSI in males from both caves (CLA and CdA). Such a pattern is traditionally interpreted as a response to reduced sperm competition (Stockley *et al.*, 1997; Pilastro *et al.*, 2002; Evans *et al.*, 2003) and parallels the reduced sperm count reported in CdA males by Franssen *et al.* (2008). Given that population densities in extreme habitats are not lower than those in benign habitats and sex ratios may even be slightly male-biased (Plath & Tobler, 2010), we interpret the observed pattern as a direct energy-saving response

to the energetically demanding environment coupled with a lack of visual orientation (for food finding) in the two caves. The lack of aggressive male–male competition, male dominance hierarchies and the reduction in frequency of male sexual behaviours in the CdA are in line with this interpretation (e.g. Plath *et al.*, 2003; Plath, 2008). Reduced sperm counts, reduced expression of colour and male sexual behaviour have also been documented in fish from waters polluted by anthropogenic sources (Toft & Guillette, 2005; Arellano-Aguilar & Macías Garcia, 2008). Then again, a reduction in sperm count, sexual behaviours and male–male aggression has also been reported for EA (Plath *et al.*, 2003; Franssen *et al.*, 2008), but GSI is even higher in EA than in RGU. At present, we do not have a compelling explanation for this conflicting pattern. Potentially, there are some – as yet unidentified – differences between EA and the other habitats that can account for this. Future research will shed light on this particular conundrum.

Local adaptation or phenotypic plasticity?

Even though males from CLA and EA were considerably smaller than CdA and RGU males in natural populations, male size more or less converged to similar size ranges in the laboratory. Even though our results do not rule out a genetic component to male size, they suggest that differences in male size are largely plastic traits shaped by environmental constraints, and possibly social cues (e.g. Travis *et al.*, 1989).

Other life-history traits, however, were highly conservative, which points to genetically based differences because of local adaptation. Fat content, for example, was significantly lower in males from all extreme habitats (CdA, CLA and EA), even when fish had been reared under benign common garden conditions for several generations. Usually, cave organisms are characterized by low metabolic rates compared to surface-dwelling relatives, and this is thought to be an adaptation to the low energy availability in most caves (review in Hüppop, 2000). In a recent study, we were able to demonstrate that this is not true for *P. mexicana* from CdA, which are actually characterized by higher metabolic rates compared to surface-dwelling *P. mexicana* (mass-specific routine metabolic rates: CdA = 0.468 ± 0.238 mL O_2 h^{-1} g^{-1}; RGU = 0.287 ± 0.124 mL O_2 h^{-1} g^{-1}; Riesch *et al.*, submitted). We propose that physiological mechanisms of (oxidative) sulphide detoxification require large amounts of energy (ATP) and oxygen, leading to higher metabolic rates. Other species that are capable of sulphide oxidation as a means of detoxification also exhibit increased metabolic rates (Grieshaber & Völkel, 1998). As CdA have higher metabolic rates even after being raised for several generations under nontoxic conditions in the laboratory (Riesch *et al.*, submitted), this indicates that the physiological adaptations to sulphide are genetically fixed and (at least to a large extent) nonplastic. Thus,

extremophile fish still consume a large amount of the available energy even in the H$_2$S-free laboratory environment, which effectively reduces the amount of excess nutrients that can be stored as body fat. In fact, this is also the most likely reason for the highly reduced size-specific lean weight exhibited by both wild-caught and laboratory-reared CdA males.

All males had even less body fat when reared under common garden conditions than when collected from the wild, and this effect was particularly strong in cave mollies (CLA and CdA). Even though major differences between populations in sexual activity have a heritable component (Plath, 2008), laboratory-reared fish are consistently more sexually active (Köhler *et al.*, 2011). This increase in sexual activity under laboratory conditions could account, in part, for the observed decrease in fat content in common garden–reared males from all populations, because increased sexual activity leads to a decrease in the time available for resource acquisition (Köhler *et al.*, 2011) as well as higher overall energy expenditure.

Why is fat in laboratory-reared males from the two cave populations (CdA and CLA) more strongly reduced than in surface males? Cave mollies not only experienced a general change from field to laboratory conditions with regard to resource availability and social environment, but for them this switch was also accompanied by a change in light conditions (i.e. permanent darkness in the field vs. a natural light/dark cycle in the greenhouse). Previous research has shown that cave mollies in light show far more sexual behaviours than in darkness (Plath *et al.*, 2004a). Furthermore, cave mollies are unpigmented in their natural environment (Gordon & Rosen, 1962), but recover much of their pigmentation after 2–3 months of exposure to UV light (R. Riesch, M. Plath & I. Schlupp, pers. observ.). All these factors constitute additional physiological costs for cave mollies in laboratory conditions that are absent in natural populations.

Conclusions

Extremophile poeciliids live in permanently stressful environments, which is predicted to lead to evolutionary change as a result of local adaptation (e.g. Calow, 1989; Gray, 1989; Sibly & Calow, 1989). Here, for the first time, we are able to provide evidence for strong male life-history divergence among locally adapted populations of *P. mexicana* from four different habitat types. Surprisingly, this differentiation was only slightly weakened in laboratory-reared males after several generations in common garden conditions. As traits such as fat content and GSI are unlikely to be heritable *per se*, this conservatism of male life-history traits suggests a different mechanism. The unpredictability of most male life histories in response to toxicity and darkness suggests that selection of these extreme environmental factors primarily acts on the offspring size/offspring number trade-off in females

(Riesch *et al.*, 2010a–c), and not in similar strength on traits such as standard length, lean weight, or gonadal investment in males. We propose that conservatism of certain male life histories is an indicator of other (heritable) changes between populations in physiology (Riesch *et al.*, submitted) and behaviour (Plath, 2008). Together with the previously demonstrated genetic (Plath *et al.*, 2007a; Tobler *et al.*, 2008a, 2009a) and morphological divergence (Parzefall, 2001; Tobler *et al.*, 2008a), this is further evidence for incipient ecological speciation in extremophile *P. mexicana*. In particular, it demonstrates the continuum of the relative strength of local adaptation in this system: fish from the most extreme habitat (the toxic cave, CdA) clearly show the strongest evolutionary divergence in traits essential to life in a permanently extreme habitat, whereas the results for CLA (dark/non-toxic) and EA (surface/toxic) indicate a relatively stronger component of phenotypic plasticity.

Acknowledgments

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