

Toxic hydrogen sulphide shapes brain anatomy: a comparative study of sulphide-adapted ecotypes in the *Poecilia mexicana* complex

T. Schulz-Mirbach¹, C. Eifert², R. Riesch³, M. S. Farnworth⁴, C. Zimmer², D. Bierbach⁵, S. Klaus², M. Tobler⁶, B. Streit², J. R. Indy⁷, L. Arias-Rodriguez⁷ & M. Plath⁸

¹ Department Biology II, Ludwig-Maximilians-University Munich, Planegg-Martinsried, Germany

² Ecology and Evolution, J. W. Goethe University Frankfurt, Frankfurt am Main, Germany

³ School of Biological Sciences, Royal Holloway, University of London, Egham, UK

⁴ Göttingen Center for Molecular Biosciences, Georg-August-University Göttingen, Göttingen, Germany

⁵ Department of Biology and Ecology of Fishes, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

⁶ Division of Biology, Kansas State University, Manhattan, KS, USA

⁷ División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco (UJAT), Villahermosa, Tabasco, México

⁸ College of Animal Science and Technology, Northwest A&F University, Yangling, China

Keywords

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Correspondence

Tanja Schulz-Mirbach, Department Biology II, Ludwig-Maximilians-University Munich, Großhaderner Straße 2, D-82152 Planegg-Martinsried, Germany.
Email: schulz-mirbach@biologie.uni-muenchen.de

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Abstract

The teleost brain is an energetically costly organ, which raises the question of how brain anatomy is shaped by divergent ecological factors in contrasting (extreme/resource-limited vs. benign) environments. A previous study compared different ecotypes of the teleost *Poecilia mexicana* in the Tacotalpa drainage system and found that cave fish had a smaller eye diameter, a smaller optic tectum and larger telencephalic lobes relative to ancestral surface-dwelling fish. Smaller eyes and a smaller optic tectum but larger telencephalic lobes were also found in fish from a sulphidic surface habitat near one of the caves, which the authors hypothesized to result from limited vision in turbid sulphide waters. In this study, we tested if repeated transitions along a replicated, natural toxicity gradient result in repeated ('convergent') anatomical changes of the teleost brain. We compared ecotypes in the *P. mexicana* species complex that have independently evolved increased tolerance to hydrogen sulphide (H₂S) in three river drainages in southern Mexico, including a phylogenetically old H₂S-adapted form (*P. sulphuraria*) and two *P. mexicana* ecotypes that represent earlier stages of adaptation to H₂S. All H₂S-adapted ecotypes exhibited smaller eyes, a smaller optic tectum volume and a smaller brain volume, but larger corpora cerebelli and hypothalamic volume than fish from non-sulphidic habitats. Drainage-specific effects were found for the telencephalic lobes, the total brain and eye size, as sexes responded differently to the presence of H₂S depending on the drainage of origin. Turbidity and toxicity in sulphidic habitats may explain patterns of brain size divergence similar in direction (but not degree) to those observed in cave ecotypes. Hence, variation in brain anatomy reflects major ecological differences, and repeated ecological gradients can result in convergent differences in brain anatomy. Nonetheless, some unique patterns of brain differentiation suggest as yet unidentified differences in selection regimes between different sulphidic springs.

Introduction

Explaining the diversity of vertebrate brain size is a major goal of evolutionary neurobiology (Isler & van Schaik, 2006, 2014; Kotrschal *et al.*, 2013). Several attempts have been made to identify the selective forces underlying inter- and intra-specific variation in brain size and the dimensions of different brain parts. The observed size variation of the brain (or single brain

parts) has been interpreted as an adaptive evolutionary response to divergent ecological and social selection in diverse groups including mammals (e.g. Harvey, Clutton-Brock & Mace, 1980; Dunbar & Shultz, 2007), birds (e.g. Sol *et al.*, 2005; Maklakov *et al.*, 2011) and teleost fishes (e.g. Gonzalez-Voyer, Winberg & Kolm, 2009; Gonzalez-Voyer & Kolm, 2010). Different, not mutually exclusive hypotheses can explain the evolution of larger brains. While the 'social brain

hypothesis' (see Dunbar & Shultz, 2007) and the 'clever-foraging hypothesis' (Striedter, 2005) focus on the benefits of increased brain size, the 'expensive tissue hypothesis' (Aiello & Wheeler, 1995; Isler & van Schaik, 2009; Tsuboi *et al.*, 2014) emphasizes the costs of maintaining this energetically costly organ. In essence, increased encephalization occurs at the expense of other energetically demanding organs resulting, for example, in a size reduction of the gut or the gonads (Aiello & Wheeler, 1995; Isler & van Schaik, 2009), and this trade-off should be especially pronounced under extreme environmental conditions.

Testing the effects of extreme ecological conditions on brain size divergence is especially promising in systems with evolutionarily replicated extreme selective forces. Until now, only few studies have tackled the question of how repeated transitions along the same ecological gradient affect brain anatomy (see Chapman, 2015; Eifert *et al.*, 2015). Eifert *et al.* (2015) provided one such example (in this case: two transitions towards cave-dwelling). Compared to fish from a benign surface habitat, both cave forms of the neotropical freshwater fish *Poecilia mexicana* were characterized by a smaller eye diameter and a smaller optic tectum, but larger telencephalic lobes. Common-garden reared individuals of both ecotypes (surface and cave forms), however, showed very similar brain proportions, highlighting the important role of developmental plasticity rather than evolved differences as a driver of the observed anatomical differences (Eifert *et al.*, 2015; for developmental plasticity of the teleost brain, see also Crispo & Chapman, 2010; Ebbesson & Braithwaite, 2012; Gonda, Herczeg & Merilä, 2013; Noreikiene *et al.*, 2015). Our present study was motivated by another result of that investigation; unlike in fish from the benign surface habitat but similar to the cave forms, smaller eyes and a smaller optic tectum, and larger telencephalic lobes were also found in fish inhabiting a surface habitat in proximity to one of the caves that contains high and sustained concentrations of toxic hydrogen sulphide (H_2S ; Gordon & Rosen, 1962; Plath & Tobler, 2010). *Poecilia mexicana* has independently colonized spring complexes with naturally occurring H_2S in at least four drainage systems (Palacios *et al.*, 2013; Plath *et al.*, 2013; Riesch, Tobler & Plath, 2015). While the study by Eifert *et al.* (2015) focused on ecologically divergent populations within the Tacotalpa drainage, we build on these findings quantifying brain gross anatomy as a response to a repeated ecological gradient of H_2S toxicity in three of the four drainage systems. The studied populations included a phylogenetically old H_2S -adapted form (described as *P. sulphuraria*) and two *P. mexicana* ecotypes that represent earlier stages of adaptation to H_2S (Palacios *et al.*, 2013; Plath *et al.*, 2013; Pfenninger *et al.*, 2014). Specifically, we compared eye diameter as well as volumes of the optic tectum, telencephalic lobes, corpus cerebelli, the hypothalamus and the whole brain between sulphide and non-sulphide populations. Convergent phenotypes are often, but not always, the result of convergent developmental modes (Wray, 2002; e.g. larger heads in extremophile *Poecilia*; Riesch *et al.*, 2011). The repeated ecological gradient in our study system allows the investigation of shared ('convergent') and unique patterns of divergence (see Langerhans & DeWitt, 2004; Langerhans & Riesch, 2013). Given the

results of Eifert *et al.* (2015), we expect shared features of divergent gross brain anatomy in *Poecilia* from H_2S toxic springs to be at least in part the result of developmental plasticity that can be induced by exposure to environmental cues (i.e. phenotypic plasticity; see Gonda *et al.*, 2013; Wiens, Crispo & Chapman, 2014).

In aquatic habitats, naturally occurring H_2S is an environmental stressor that is toxic in micromolar amounts for most metazoans (Riesch *et al.*, 2015). Adverse effects of H_2S on organisms are twofold: sulphide directly hampers respiratory chain functioning in mitochondria and blood oxygen uptake (Pfenninger *et al.*, 2014; Riesch *et al.*, 2015); at the same time, oxidation of H_2S creates severe hypoxia in the water. Despite its adverse effects, several fishes – including populations of the *P. mexicana* species complex – have adapted to these harsh environmental conditions (Riesch *et al.*, 2015), resulting in distinct phenotypic changes including physiology (e.g. altered metabolic rates; Passow *et al.*, 2015), morphology (increased head and gill size; Tobler *et al.*, 2011; Palacios *et al.*, 2013) and life-history traits (fewer but larger offspring; Riesch *et al.*, 2014, 2015). To cope with hypoxia, poeciliids in sulphidic habitats exploit the more oxygen-rich topmost layer of the water column by performing aquatic surface respiration (ASR; Brauner *et al.*, 1995; Plath *et al.*, 2007b). Because sulphide-adapted fishes spend a considerable amount of their time budget engaging in ASR, less time is available for foraging, as indicated by behavioural observations and analyses of gut fullness (Tobler *et al.*, 2009). Low food (i.e. energy) availability and energetically demanding H_2S detoxification (cf. Plath *et al.*, 2007b; Tobler *et al.*, 2009; Passow *et al.*, 2015) consequently impose constraints on individual energy budgets, and theory predicts that this should exert selection on reduced energy demands. This can be accomplished through reductions in mass-specific metabolic rates and body size (Passow *et al.*, 2015). In addition, selection is predicted to reduce the dimensions of energetically demanding organs (such as the brain or parts thereof). The African cichlid *Pseudocrenilabrus multicolor victoriae* inhabiting both normoxic and hypoxic waters, for example, showed reduced brain mass when fish were experimentally reared under low dissolved oxygen concentrations (Crispo & Chapman, 2010; Wiens *et al.*, 2014). One component of the brain that may be reduced in sulphidic habitats, as a result of the increased turbidity induced by precipitating sulphur compounds (Bagarinao, 1992), is the optic tectum. Increased turbidity may limit visual orientation and communication in sulphidic habitats, and visual input is a trigger of optic tectum growth (Ishikawa *et al.*, 1999, 2001; Soares *et al.*, 2004). Indeed, sulphide-adapted fish have significantly smaller eyes than fish from non-sulphidic habitats in at least one sulphide spring complex (Plath *et al.*, 2007a; Eifert *et al.*, 2015).

Based on the nature of eco-physiological stressors outlined above (sulphide toxicity and resource-limitation, coupled with reduced visibility) and some similarities to cave environments (Eifert *et al.*, 2015), we predicted to find smaller brains, smaller optic tecta and smaller eyes in populations from sulphidic waters compared to those from non-sulphidic habitats. Due to an increased importance of non-visual senses (chemical senses) in turbid waters (Kotrschal, Van Staaden & Huber, 1998), we

further predicted larger telencephalic lobes, which receive olfactory and gustatory input (Bshary, Gingins & Vail, 2014). In addition, we expected the corpus cerebelli to be larger in sulphide-adapted fishes, as the cerebellum is responsible for motor coordination and body orientation (Kotrschal *et al.*, 1998; Broglio, Rodríguez & Salas, 2003; Butler & Hodos, 2005) and thus might play an important role for fishes to better orient themselves and avoid predators in turbid waters (Tobler, 2009; Riesch *et al.*, 2010) by improved mechanosensation.

Materials and methods

Study system and sample collection

The occurrence of sulphidic waters in south-east Mexico is likely associated with the activity of the El Chichón Volcano (Rosales Lagarde *et al.*, 2006). The sampled sulphide spring complexes are located in the foothills of the Sierra Madre de Chiapas and are distributed across the three drainage systems Río Tacotalpa (Tac), Río Puyacatengo (Puy) and Río Pichucalco (Pich; Fig. 1a). All three drainages eventually join the Río Grijalva and are widely interconnected in the lowlands during the wet season. The sulphide spring complexes, however, are permanently separated by mountains (Miller, 1966). Within each drainage system, non-sulphidic and sulphidic sites are interconnected and not separated by physical barriers that would prevent fish migration. Average sulphide concentrations in the spring systems range between 23 and 190 μM (Tobler *et al.*, 2006, 2011). In addition, sulphidic habitats exhibit higher temperatures and higher specific conductivities as well as lower pH and lower dissolved oxygen concentrations than non-sulphidic habitats (Tobler *et al.*, 2011).

Ecotypes of the *P. mexicana* species complex have independently evolved resistance to naturally occurring hydrogen sulphide (H_2S) in the three river drainages, including a phylogenetically old H_2S -adapted form (described as *P. sulphuraria* Álvarez, 1948) and two ecotypes of *P. mexicana* Steindachner, 1863 that represent earlier stages of adaptation to H_2S (Pfenninger *et al.*, 2014). *Poecilia sulphuraria* is endemic to the Baños del Azufre and other sulphide spring complexes in Pich (Tobler & Plath, 2009). The closest relative of *P. sulphuraria* presumably went extinct in this area, such that *P. sulphuraria* is more closely related to (northeast Mexican) *P. mexicana limantouri* (Palacios *et al.*, 2013). We thus relied on a comparison of a population of *P. mexicana mexicana* from a nearby non-sulphidic site, as we did not have access to wild-caught *P. mexicana limantouri* specimens (Pich-1, Table 1). To demonstrate that this comparison was still biologically meaningful and not flawed by phylogenetic distance, we included another, even more distantly related species (Peten molly, *P. petenensis* Günther, 1866) from a non-sulphidic habitat in the same drainage (Pich-2, Table 1). In addition, we included one *P. mexicana* population each from sulphidic and adjacent non-sulphidic sites in Tac and Puy.

Immediately after capture, all fishes ($N_{\text{females}} = 47$, $N_{\text{males}} = 41$; see Table 1) were anaesthetized and euthanized using an overdose of buffered tricaine methanesulfonate (MS-

222; Sandoz, Switzerland, Bern) and preserved in 70% ethanol.

Preparation and measurements of brains and eyes

We measured standard length and eye size (maximum diameter) of ethanol-preserved specimens with a sliding calliper to the nearest 0.05 mm (Supporting Information Table S1). All samples were then rehydrated through a descending ethanol series (50%, 30%, deionized water) for 1 h at each step and fixed in a 10% formalin solution for 1 week. Prior to dissection, heads were separated and soaked in deionized water for 1 h. Brains were then separated from the spinal cord in the region of the medulla oblongata, dissected out and stored in 10% formalin solution. Dissected brains were placed in a glass cup with 0.1% agarose gel and photographed through a stereo-microscope camera (Nikon DS-Fi1, Nikon Corp., Tokyo, Japan) in lateral, ventral and dorsal projections, after which they were weighed to the closest 0.01 g using a micro balance (Sartorius 4505 MP6; Sartorius AG, Göttingen, Germany; accuracy 0.001 mg).

We measured the length, width and height of the telencephalic lobes, optic tectum, corpus cerebelli, hypothalamus and total brain (Fig. 1b) with NIS-Elements BR 3.2 (Nikon Corp.). Measurements followed the protocol described by Pollen *et al.* (2007). We determined total brain length from the anterior part of the telencephalic lobes to the caudal part of the corpus cerebelli while excluding the medulla oblongata. Mean values of measured widths of both hemispheres of the telencephalic lobes, optic tecta and hypothalami were used for statistical analysis. We estimated brain region volumes and total brain volume using the ellipsoid volume formulas (e.g. Pollen *et al.*, 2007):

$$\text{Brain region volume} = (\text{length} \times \text{width} \times \text{height}) \times \frac{\pi}{6}.$$

Total brain volume

$$= (\text{total brain length} \times \text{total brain width} \times \text{total brain height}) \times \frac{\pi}{6}.$$

Statistical analyses

To correct for size differences between individuals, we included a covariate in all statistical models. The covariate for size correction of brain (region) volumes was derived from a correlation matrix-based principal component analysis (PCA) on the variables 'standard length' and 'brain mass' (Eifert *et al.*, 2015). Principal component (PC) 1 explained 90.13% of the total variance and 'standard length' and 'brain mass' each had axis loadings of 0.949 on the first PC. Eye diameter was size corrected using standard length as a covariate. The factor 'sex' was included in all statistical models because of the well-known sexual dimorphism of the teleost brain (Bass & Grober, 2001; Kotrschal *et al.*, 2012).

We conducted two analyses: Our first approach tested for general patterns of brain region size divergence based on habitat

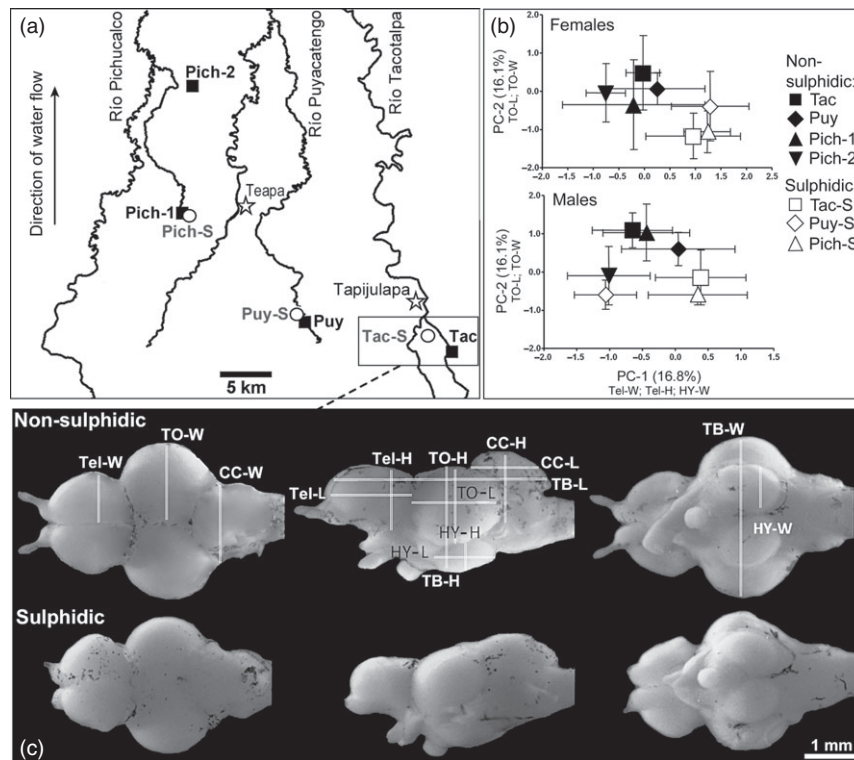


Figure 1 (a) Study area near the city of Teapa in southern Mexico showing the three drainage systems and sample sites. Open circles indicate sulphidic (Pich-S; Puy-S and Tac-S) and black squares non-sulphidic sites (Pich-1; Pich-2; Puy; Tac); for site abbreviations see Table 1. (b) Brain measurements for representative brains of fish from non-sulphidic (Tac) and sulphidic (Tac-S) habitats (*P. mexicana*; Río Tacotalpa drainage) in dorsal (left column), lateral (middle) and ventral (right) projections. Anterior is to the left. (c) Scatter plot showing the separation of sulphide-adapted *Poecilia* spp. and fish from non-sulphidic sites based on mean (\pm SD) residual principal component scores (corrected for allometric size effects using a preparatory MANCOVA; per cent variance explained is given in parentheses) of brain dimensions (see Supporting Information Tables S1 and S2). For clarity of illustration, females (above) and males (below) are shown in separate plots based on a single PCA performed with data from both sexes combined. Measurements: L, length; H, height; W, width; brain regions: CC, corpus cerebelli; HY, hypothalamus; Tel, telencephalic lobes; TB, total brain; TO, optic tectum.

Table 1 Overview of the studied populations of *Poecilia* spp. from different sites in the Río Pichucalco, Río Puyacatengo and Río Tacotalpa drainages in the states of Tabasco and Chiapas in southern Mexico, including site abbreviations (Site ID), sample sizes (*N*, males/females) and occurrence of toxic hydrogen sulphide (H₂S)

Species	Site (lab code)	Site ID	H ₂ S	Latitude	Longitude	<i>N</i> (♂/♀)
Río Pichucalco drainage						
<i>P. mexicana</i>	Río Pichucalco	Pich-1	–	17.55225	–92.99859	12 (5/7)
<i>P. petenensis</i>	Lagoon West 195	Pich-2	–	17.67906	–92.99824	11 (6/5)
<i>P. sulphuraria</i>	Baños del Azufre	Pich-S	+	17.55225	–92.99859	11 (7/4)
Río Puyacatengo drainage						
<i>P. mexicana</i>	Río Puyacatengo	Puy	–	17.45512	–92.88768	12 (3/9)
<i>P. mexicana</i>	La Luvia, small spring	Puy-S	+	17.46387	–92.89541	15 (8/7)
Río Tacotalpa drainage						
<i>P. mexicana</i>	Arroyo Bonita	Tac	–	17.42685	–92.75213	10 (4/6)
<i>P. mexicana</i>	El Azufre	Tac-S	+	17.44225	–92.77447	17 (8/9)

types. For this analysis, lengths, widths and heights of brain regions (telencephalic lobes, optic tectum, hypothalamus, corpus cerebelli) were size-corrected with the combined covariate in a preparatory MANCOVA, and residuals were subjected to a PCA based on a correlation matrix using the varimax method. In

addition to the six *P. mexicana*/*P. sulphuraria* populations, the PCA included *P. petenensis* to illustrate potential effects of toxicity on brain size also between distantly related taxa.

Second, we performed separate ANCOVAs for the six *P. mexicana*/*P. sulphuraria* populations (excluding *P. petenensis*)

using eye diameter and brain (region) volumes as dependent variables, 'drainage', 'toxicity' and 'sex' as fixed factors and standard length (for eye size) or the combined PC (for brain region volumes) as the covariate. We included all interaction terms between fixed factors (two- and three-way interactions) and all two-way interactions between fixed factors and the covariate. As all interaction terms between fixed factors and the covariate were not significant (except for the analysis of hypothalamus volume), we omitted these interactions from the final models. The analysis of hypothalamus volume indicated significant effects of 'sex \times covariate' ($F_{1,60} = 6.99$, $P = 0.010$, $\eta_p^2 = 0.10$) and 'drainage \times covariate' ($F_{2,60} = 3.61$, $P = 0.033$, $\eta_p^2 = 0.11$); these two interactions were therefore retained in the final model for the analysis of this brain region. The other final models included all main effects, all two-way interactions between the fixed factors and the three-way interaction 'drainage \times toxicity \times sex'. In the final ANCOVAs, main effects of 'toxicity' indicate shared responses to the presence of H₂S across drainages, while interaction effects involving 'toxicity' and 'drainage' indicate unique (drainage-specific) effects.

Dependent variables that did not meet the assumptions of homogeneity of variances and normal distribution were ln-transformed. Ln-transformed variables met the assumptions of homogeneity of variances and normal distribution. We interpreted significant results as biologically meaningful based on relative effect sizes (partial eta squared, $\eta_p^2 > 0.1$), which provide a measure of the explanatory ability of a factor relative to unexplained variation (for details refer to Langerhans & DeWitt, 2004). PCAs were performed using SPSS 13.0 (SPSS Inc., Chicago, IL) and ANCOVAs using PASW 18.0 (IBM SPSS Statistics).

Results

Differences between ecotypes based on brain region measurements (PCA)

The first five PCs exhibited eigenvalues >1 and explained 68.40% of the total variance. Measurements of all four brain regions largely contributed to the first five PCs ($|\text{loading}| \geq 0.625$; Supporting Information Table S2). Visual inspection of population separation along PC axes 1 and 2 (Fig. 1c) suggested two clearly distinguishable clusters (sulphide and non-sulphide populations), and the distantly related *P. petenensis* clustered among *P. mexicana* populations from non-sulphidic habitats. The separation was particularly clear-cut in females, whereas males from the contrasting habitats were less separated mainly due to the small values of Puy-S males along PC 1, that is, these males displayed a smaller width and height of the telencephalic lobes and smaller hypothalamus width than males from Tac-S and Pich-S.

Along PC axis 1, female *Poecilia* spp. from sulphidic habitats were separated from females originating from non-sulphidic habitats by the height and width of telencephalic lobes and the height of the hypothalamus (Fig. 1c). Along PC axis 2, separation between the two habitat types in both

sexes was mainly driven by differences in length and width of the optic tectum (Fig. 1c).

Differences between ecotypes in brain (region) volumes and eye size (ANCOVAs)

The proxy for body size – that is, the covariate standard length for eye diameter and the combined covariate for total brain volume and brain region volumes – had the strongest effect on brain (region) volumes and eye diameter in all ANCOVAs ($\eta_p^2 \geq 0.544$; Table 2), indicating that these traits increased with increasing body size. The factor 'toxicity' (representing the shared/'convergent' component of trait divergence in our analyses) had a significant effect on the volume of the optic tectum, the corpus cerebelli, the hypothalamus and the total brain, as well as on eye diameter ($P \leq 0.008$, $\eta_p^2 \geq 0.108$). Fishes from sulphidic habitats had significantly larger corpora cerebelli and hypothalami, while the volume of the optic tectum, the volume of the total brain and the eye diameter were smaller than in fish from non-sulphidic habitats (Figs 2c, e, 3a, d, 4a). Sex- and drainage-specific patterns of divergence are described for each trait next.

Telencephalic lobes

The factor 'sex' had a significant effect on the size of telencephalic lobes ($P < 0.001$, $\eta_p^2 = 0.217$), with females possessing larger lobes than males (Fig. 2a). The interaction term 'drainage \times toxicity \times sex' suggests drainage-specific differences in how this sexual dimorphism was affected by the presence of H₂S ($P = 0.008$, $\eta_p^2 = 0.139$). While telencephalic lobes of both sexes were either larger (Tac) or smaller (Pich) in sulphidic habitats, a different pattern was observed in the Puy (Fig. 2b): Puyacatengo females from the sulphidic site had smaller telencephalic lobes than those from the non-sulphidic site, while sulphide-adapted males showed distinctly larger telencephalic lobes than males from the benign habitat (Fig. 2b).

Optic tectum and eye diameter

Sexual dimorphism in the volume of the optic tectum varied between drainages (effect of 'drainage \times sex'; $P = 0.033$, $\eta_p^2 = 0.101$). Females had smaller optic tecta than males in Tac and Pich, but larger optic tecta in Puy (Fig. 2d). Regarding eye diameter, the interaction 'drainage \times toxicity \times sex' indicated drainage-specific differences in how sexual dimorphism was affected by the presence of H₂S ($P = 0.006$, $\eta_p^2 = 0.148$). In two drainages, sex differences were absent or small, while females from the non-sulphidic site in Pich had distinctly larger eyes than males (Fig. 2f).

Corpus cerebelli

The factor 'drainage' significantly affected the size of the corpus cerebelli ($P < 0.001$, $\eta_p^2 = 0.259$). Corpora cerebelli were smallest in fish from Tac and largest in those from Pich (Fig. 3b). The interaction 'drainage \times toxicity' indicated

Table 2 Results of ANCOVAs testing the effect of H₂S toxicity on brain (region) volumes and eye size in ecotypes of the *P. mexicana* complex [Pichucalco: Pich-1, Pich-S (*P. sulphuraria*); Puyacatengo: Puy, Puy-S; Tacotalpa: Tac, Tac-S; see Table 1]

Source	Effect	d.f.	<i>F</i>	<i>P</i>	η_p^2
Telencephalic lobes	Covariate (SL, brain mass)	1	110.289	<0.001	0.633
	Drainage	2	2.012	0.142	0.059
	Toxicity	1	1.935	0.169	0.029
	Sex	1	17.784	<0.001	0.217
	Drainage × toxicity	2	3.242	0.046	0.092
	Drainage × sex	2	2.491	0.091	0.072
	Toxicity × sex	1	6.192	0.015	0.088
	Drainage × toxicity × sex	2	5.178	0.008	0.139
	Error	64			
Optic tectum	Covariate (SL, brain mass)	1	106.954	<0.001	0.626
	Drainage	2	1.039	0.360	0.031
	Toxicity	1	31.897	<0.001	0.333
	Sex	1	0.925	0.340	0.014
	Drainage × toxicity	2	1.001	0.373	0.030
	Drainage × sex	2	3.598	0.033	0.101
	Toxicity × sex	1	3.561	0.064	0.053
	Drainage × toxicity × sex	2	1.300	0.280	0.039
	Error	64			
Corpus cerebelli	Covariate (SL, brain mass)	1	117.544	<0.001	0.647
	Drainage	2	11.202	<0.001	0.259
	Toxicity	1	22.329	<0.001	0.259
	Sex	1	0.119	0.731	0.002
	Drainage × toxicity	2	5.021	0.009	0.136
	Drainage × sex	2	0.800	0.454	0.024
	Toxicity × sex	1	1.125	0.293	0.017
	Drainage × toxicity × sex	2	0.716	0.493	0.022
	Error	64			
Hypothalamus	Covariate (SL, brain mass)	1	72.664	<0.001	0.544
	Drainage	2	4.103	0.021	0.119
	Toxicity	1	7.405	0.008	0.108
	Sex	1	0.121	0.729	0.002
	Drainage × toxicity	2	1.384	0.258	0.043
	Drainage × sex	2	0.090	0.914	0.003
	Toxicity × sex	1	0.843	0.362	0.014
	Drainage × covariate	2	0.902	0.411	0.029
	Sex × covariate	1	6.474	0.013	0.096
	Drainage × toxicity × sex	2	0.783	0.462	0.025
	Error	61			
Total brain	Covariate (SL, brain mass)	1	215.682	<0.001	0.771
	Drainage	2	0.994	0.376	0.030
	Toxicity	1	8.711	0.004	0.120
	Sex	1	0.968	0.329	0.015
	Drainage × toxicity	2	1.058	0.353	0.032
	Drainage × sex	2	5.682	0.005	0.151
	Toxicity × sex	1	0.115	0.736	0.002
	Drainage × toxicity × sex	2	8.010	0.001	0.200
	Error	64			
Eye diameter	Covariate (SL)	1	91.742	<0.001	0.589
	Drainage	2	0.258	0.773	0.008
	Toxicity	1	17.646	<0.001	0.216
	Sex	1	0.722	0.399	0.011
	Drainage × toxicity	2	0.138	0.871	0.004
	Drainage × sex	2	2.896	0.063	0.083
	Toxicity × sex	1	5.767	0.019	0.083
	Drainage × toxicity × sex	2	5.562	0.006	0.148
	Error	64			

Volumes of telencephalic lobes, optic tecta, corpora cerebelli, hypothalami and the total brain (all ln-transformed except corpus cerebelli), as well as eye diameter were used as dependent variables, and 'drainage', 'toxicity' and 'sex' as fixed factors. Significant effects are highlighted in bold.

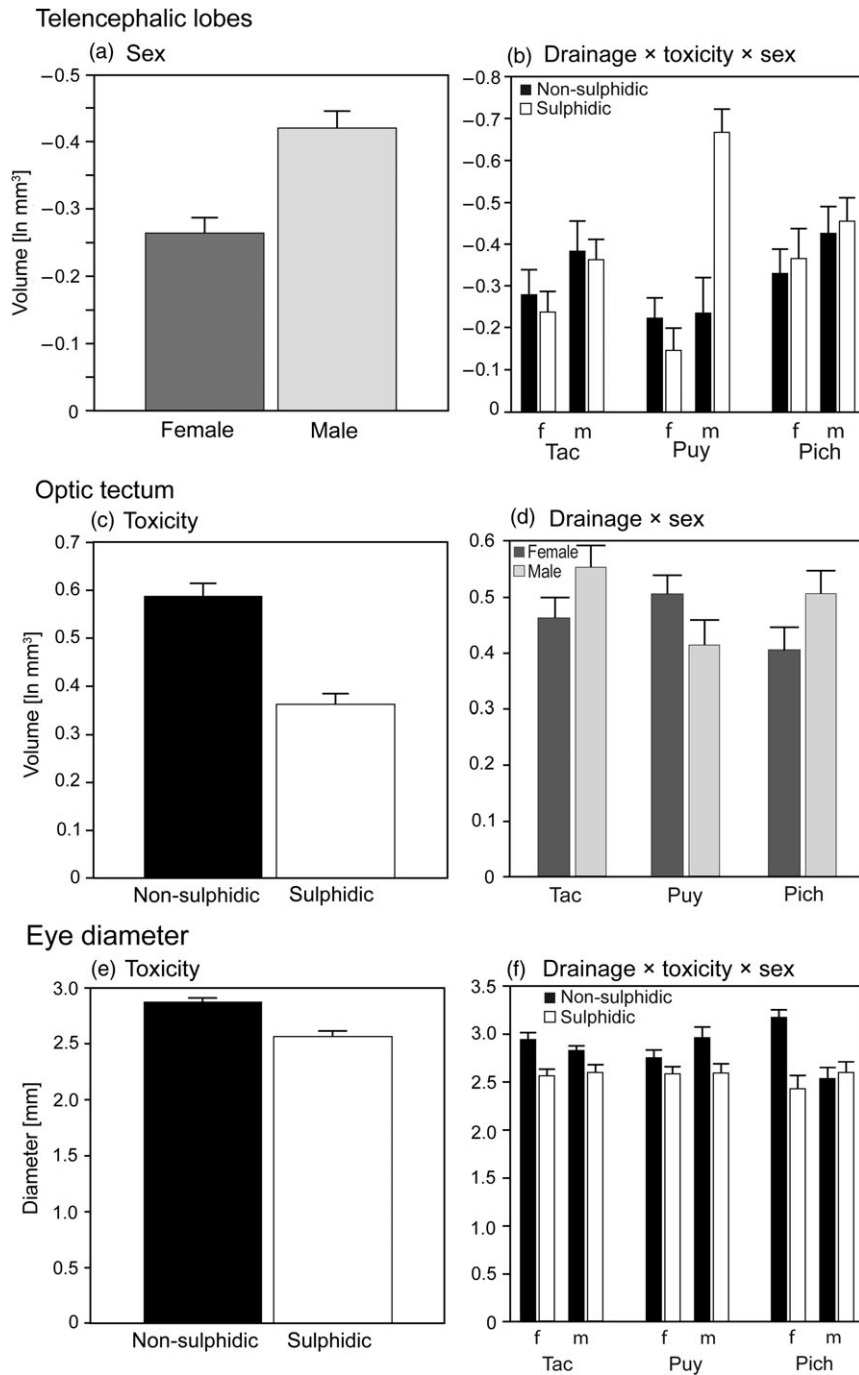


Figure 2 Mean (\pm SEM) ln-transformed volumes (estimated marginal means, EMMs) of (a and b) telencephalic lobes, (c and d) optic tectum and (e and f) mean (\pm SEM) eye diameter illustrating main toxicity effects (a, c, e) and interaction terms (b, d, f) (for P and η_p^2 values, see Table 2). Note that in (a and b) negative values are due to ln-transformation, whereby large negative values indicate smaller telencephalic lobes.

drainage-specific differences with regard to the toxicity effect ($P = 0.009$, $\eta_p^2 = 0.136$). Although sulphide-adapted fishes from all three drainages had larger corpora cerebelli than those from the normoxic habitats, habitat-specific size differences were more pronounced in Pich than in the other drainages (Fig. 3c).

Hypothalamus

The factor ‘drainage’ significantly affected the size of the hypothalamus ($P = 0.021$, $\eta_p^2 = 0.119$). The hypothalamus volume was distinctly smaller in fish from Puy than in those from Tac and Pich (Fig. 4b).

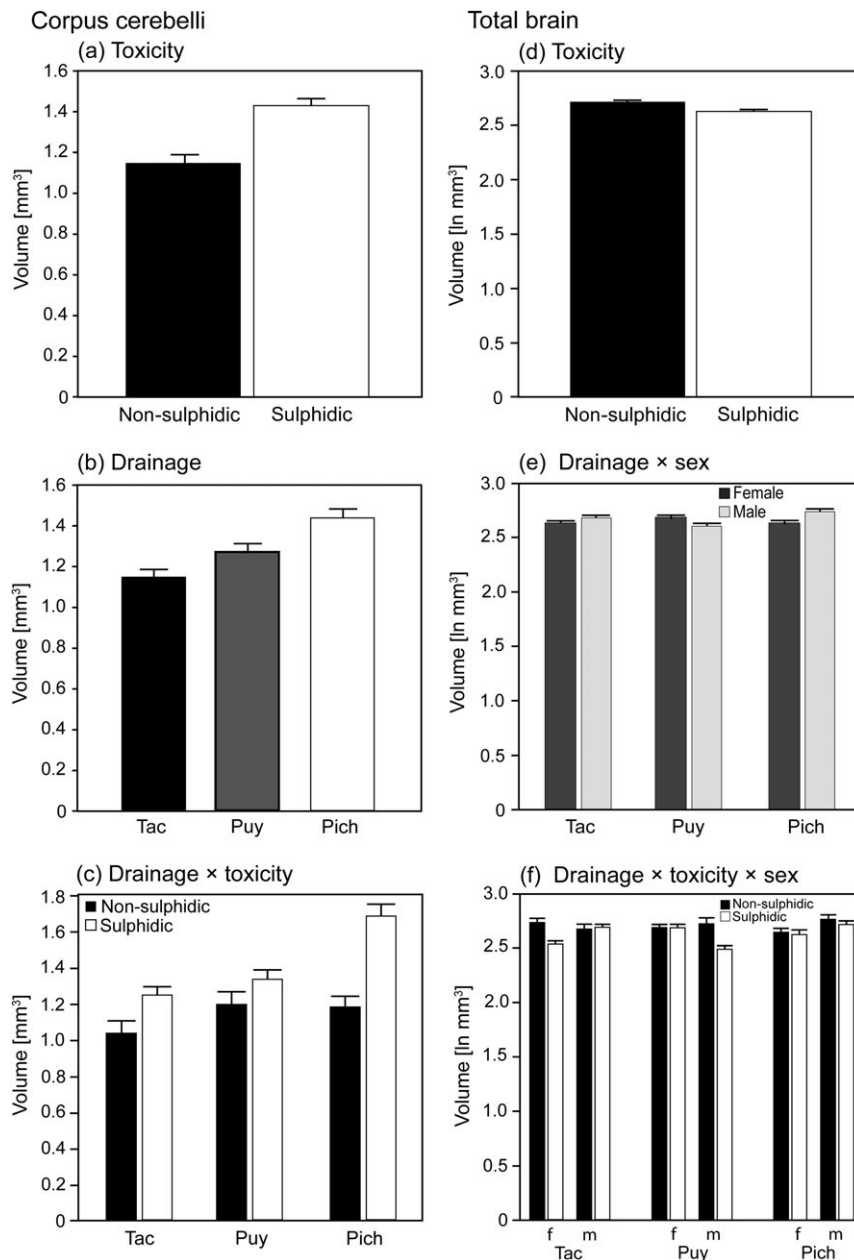


Figure 3 Mean (\pm SEM) volumes (estimated marginal means, EMMs) of the (a–c) corpus cerebelli and (d–f) total brain illustrating main toxicity effects (a, b, d) and interaction terms (c, e, f) (for P and η_p^2 values, see Table 2).

Total brain

The interaction ‘drainage \times sex’ suggests drainage-specific differences in sexual dimorphism ($P = 0.005$, $\eta_p^2 = 0.151$). Females were characterized by smaller brains than males in Tac and Pich, but larger brains in Puy (Fig. 3e; see similar drainage-specific sex differences for the optic tectum, Fig. 2d). The interaction ‘drainage \times toxicity \times sex’ further indicated drainage-specific differences in how sexual dimorphism was affected by the presence of H_2S ($P = 0.001$, $\eta_p^2 = 0.200$). In

Tac, toxicity had a distinct effect on females’ total brain size, but no effect on that of males. On the contrary, toxicity affected total brain size of males but not (or only weakly) that of females in Puy and Pich (Fig. 3f).

Discussion

In a system with replicated sulphide-adapted populations and populations in sulphide-free waters of the *P. mexicana* species complex, we asked whether brain (region) dimensions and eye

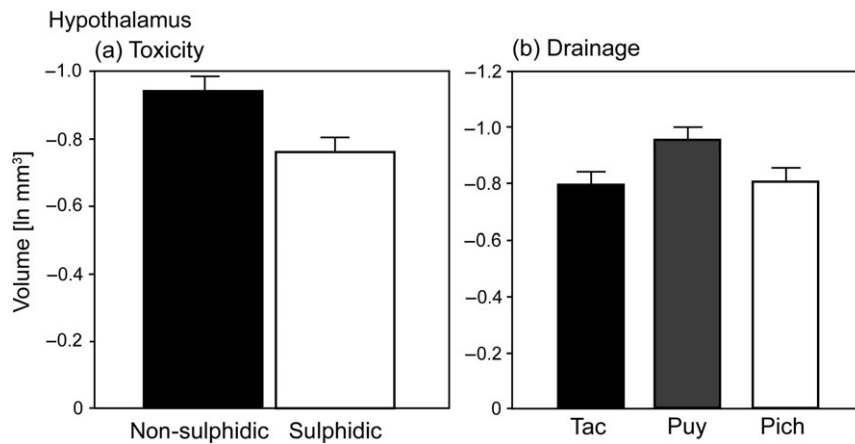


Figure 4 Mean (\pm SEM) volumes (estimated marginal means) of the hypothalamus depicting the main toxicity (a) and drainage effects (b) (for P and η_p^2 values, see Table 2). Note that negative values are due to ln-transformation, and large negative values indicate smaller hypothalamus size.

size consistently differed between habitat types irrespective of the drainage of origin. We found that habitat type – and to a lesser degree drainage system of origin and sexual dimorphism – affected the size of the brain and the size of the brain regions as well as eye diameter. Consistent with our predictions, fishes from sulphidic habitats showed a shared response to the factor toxicity across drainages by possessing a smaller volume of the optic tectum associated with a smaller eye diameter and a smaller total brain volume, but larger corpora cerebelli and hypothalamic volume. The predicted enlargement of telencephalic lobes, however, was only found in sulphide-adapted fish from one drainage (Tac).

Unique, that is, drainage-specific, effects were reflected as differences (i) in the magnitude of the population difference, but with a similar trend between fishes from sulphidic and non-sulphidic sites across drainages (corpus cerebelli), (ii) between sexes, which responded differently to H_2S depending on the drainage of origin, but again with a similar trend from sulphidic and non-sulphidic sites across drainages (total brain volume, eye size), (iii) between sexes, which responded differently to H_2S depending on the drainage of origin, but without a discernible trend across drainages (telencephalic lobes).

In the following, we will discuss the shared and unique patterns of brain (region) size variation mainly in the light of phenotypic plasticity, as heritability estimates of a previous study (Eifert *et al.*, 2015) indicated low heritability for brain (region) size in Tac. We are, however, aware that the extent of heritable components of trait divergence in the Pich system might differ from that shown for Tac (Eifert *et al.*, 2015). If the plastic responses that occur in sulphide spring environments are adaptive, it is conceivable that genetic assimilation may cause a loss of plasticity and generates heritable modifications of brain (region) size. Hence, mechanisms driving population differences (relative contributions of plastic vs. genetic variation) may vary among drainages. For example, a loss of ancestral plasticity may be most likely in the Pich drainage, where

populations have been evolving in sulphidic environments for a longer period of time (Pfenninger *et al.*, 2014).

Unique patterns of brain region size variation

We predicted larger telencephalic lobes in sulphide-adapted fishes due to an increased importance of non-visual senses (chemical sense and mechanosensation) in turbid waters (Kotrschal *et al.*, 1998). The question arises as to why the telencephalic lobes are only larger in sulphide fish from Tac. Unique responses may be explained by three main not mutually exclusive factors that relate to drainage-specific differences in (i) unique evolutionary histories of the respective populations, for example, due to genetic drift (see Pfenninger *et al.*, 2015), (ii) abiotic ecological parameters, (iii) selection from the social environment, such as differences in operational sex ratios or strength of sexual selection.

Unique plastic responses of brain regions might be explained by unique evolutionary histories of the populations (Langerhans & DeWitt, 2004; Langerhans & Riesch, 2013). A recent population genomic study comparing population pairs of sulphide-adapted versus non-sulphide fish from Tac and Puy revealed widely unique evolutionary trajectories and thus a low degree of parallelism for the adaptation to the same extreme habitat type (Pfenninger *et al.*, 2015). Mechanisms of H_2S resistance that represent a key adaptation to these extreme environments evolved in parallel only in *P. mexicana* from Puy-S and *P. sulphuraria* (Pich-S) (Pfenninger *et al.*, 2014). In both populations, parallel amino acid substitutions of the COX1 and COX3 subunits of the cytochrome-c oxidase complex (COX) lead to a less H_2S -susceptible COX, while sulphide fish from the Tac system with an H_2S -susceptible COX seem to rely on a different – yet unknown – mechanism of H_2S resistance (Pfenninger *et al.*, 2014, 2015). The question of whether differences in water chemistry between habitats (Tobler *et al.*, 2011; Palacios *et al.*, 2013) or drainage-specific

behavioural differences (Bierbach *et al.*, 2012) contribute to larger telencephalic lobes in Tac-S fish needs further investigation.

Shared patterns of brain (region) size variation

Variation of brain region sizes reflects a mosaic pattern of brain divergence

Two hypotheses – initially formulated for explaining brain size variation in mammals (e.g. Finlay & Darlington, 1995; Barton & Harvey, 2000) – have been proposed to explain the diversity in the anatomy and size of vertebrate brains. According to the mosaic evolution hypothesis, different brain regions can change independently, allowing for rapid adaptive trait divergence (Barton & Harvey, 2000). In contrast, the concerted evolution hypothesis assumes constraints to brain region evolution because of developmental interdependencies (Finlay & Darlington, 1995; Finlay, Darlington & Nicastro, 2001; Yopak *et al.*, 2010). Consistent with several previous studies (e.g. Noreikiene *et al.*, 2015; White & Brown, 2015) and a recent study on cave forms of *P. mexicana* (Eifert *et al.*, 2015), our finding of smaller optic tecta, but larger corpora cerebelli and hypothalami in sulphide-adapted *Poecilia* spp. provide additional support for a mosaic pattern of brain variation in fishes. Even though patterns of brain variation uncovered here might be plastic (Eifert *et al.*, 2015), a recent study (Noreikiene *et al.*, 2015) provided new insights into the relative roles of plasticity and heritable components of brain divergence using parent–offspring regression analysis (animal model approach). Noreikiene *et al.* (2015) demonstrated that brain region differences in the three-spined stickleback *Gasterosteus aculeatus* show low heritability estimates, and different brain regions can respond independently (in a mosaic-like manner) and plastically to varying environmental conditions (environmental enrichment treatment vs. control) even though moderate estimates for the evolvability of brain regions were uncovered. Given the mosaic pattern, the question arises what factors trigger the size reduction in the optic tectum and the enlargement of the corpus cerebelli and the hypothalamus in sulphide-adapted *Poecilia* spp.

Smaller optic tecta reflect reduced visual input and energy limitation

In cave and surface forms of the Mexican tetra *Astyanax mexicanus* (Soares *et al.*, 2004) and Medaka *Oryzias latipes* mutants (Ishikawa *et al.*, 1999, 2001), visual input, that is, the amount of light, is positively correlated with the growth and maintenance of the optic tectum. Smaller optic tecta in sulphide-adapted fishes may thus be a consequence of limited visual input in murky, turbid sulphidic waters. This assumption of a (plastic) response to turbidity is corroborated by a recent study on guppies (*P. reticulata*; Ehlman *et al.*, 2015). Fish that had been reared in turbid waters showed increased activity associated with increased gene expression of long-wavelength opsins – instead of mid-wavelength opsins – when adult

animals were tested in turbid water. In contrast, fish that did not face turbid conditions before adulthood were characterized by decreased activity.

In addition to reduced visual input as a trigger of smaller optic tectum size, the size might also have decreased to allow for larger corpora cerebelli and hypothalami in sulphide-adapted fishes. Smaller optic tecta would then be the result of a developmental trade-off due to energy limitation in sulphidic habitats (Tobler, 2008), in which maintenance of the energetically costly brain is especially challenging. Support for this idea stems from the observation that total brain sizes are also smaller in sulphide-adapted fishes than in those from benign habitats.

Larger corpora cerebelli in sulphide-adapted fishes

Habitat complexity (e.g. tanks equipped with stones and walls vs. ‘empty’ tanks) was found to be positively correlated with larger corpora cerebelli in rainbow trout *Oncorhynchus mykiss* (Kihlslinger & Nevitt, 2006) and Lake Tanganyika cichlids (Pollen *et al.*, 2007). As there is no evidence for higher habitat complexity in the sulphide sites compared to the benign habitats, this explanation is highly unlikely to account for larger corpora cerebelli in sulphide-adapted fishes.

The Daffodil cichlid *Neolamprologus pulcher* developed larger corpora cerebelli when reared in larger groups, that is, in more complex social environments (Fischer *et al.*, 2015). *Poecilia sulphuraria* that possess the largest corpora cerebelli among the sulphide-adapted fishes investigated here form dense synchronized schools as an adaptive behavioural response to increased aerial predation risk (see Riesch *et al.*, 2010). Larger size of this brain region might therefore be correlated with schooling behaviour and highly coordinated escape manoeuvres in this species, as the cerebellum plays also an important role for motor coordination and body orientation (Kotrschal *et al.*, 1998; Broglio *et al.*, 2003; Butler & Hodos, 2005).

Endogenous H₂S plays a role in fine tuning of cell signalling processes (for a review, see Li, Rose & Moore, 2011). Recently, a neuroprotective function of endogenous H₂S in the central nervous system of fishes was hypothesized (Puschina & Varaksin, 2011; Varaksin & Puschina, 2011; Sachin, Puschina & Varaksin, 2014). Although the cystathionine- γ -lyase is a key enzyme for H₂S synthesis in tissues like liver and gills (cf. Stipanuk & Ueki, 2011), endogenous H₂S is mainly produced in the brain via cystathionine- β -synthase (CBS), whereby the cerebellum is assumed to be the centre of H₂S synthesis in the fish brain (Puschina & Varaksin, 2011; Varaksin & Puschina, 2011). Surprisingly, the expression rate of the cystathionine- γ -lyase gene in liver and gill tissues did not differ between Tac fish from non-sulphidic and sulphidic habitats; when exposed to H₂S, fish from non-sulphidic habitats, however, showed a trend towards a higher expression than those from the sulphidic site (Tobler *et al.*, 2014). Nonetheless, it would be tempting to speculate that larger corpora cerebelli in sulphide-adapted fishes – especially those in *P. sulphuraria* – are correlated with the synthesis of endogenous H₂S, that is, that gene

expression rate of CBS (or CBS activity) differs between fishes from sulphidic and non-sulphidic habitats. This hypothesis, however, requires testing in future studies that also compare CBS gene expression and CBS activity in different brain regions.

Brain size variation and behaviour/cognition

As outlined above, total brains and brain regions of sulphide-adapted fishes display different (shared and unique) responses to the presence of the toxicant H₂S. Altered brain (region) sizes in sulphide fishes may be linked to observed behavioural differences between sulphide and non-sulphide fishes (see Discussion below). According to the 'ecological cognition hypothesis' (Healy & Braithwaite, 2000; White & Brown, 2015), challenging environmental conditions affect brain anatomy and behaviour, that is, brains as well as behaviour are mainly shaped by the environmental conditions and especially by the challenges these (extreme) conditions impose on the animals (White & Brown, 2015). The following discussion will focus on potential links between modified behavioural traits of sulphide-adapted fishes and the herein reported brain size variation.

Role of predation for brain size variation

Several studies indicated a correlation between brain (region) size and predation regime as well as predation avoidance behaviour (Gonda *et al.*, 2012; van der Bijl *et al.*, 2015; Kotschal *et al.*, 2015). Even though piscivorous fish species are absent from sulphidic habitats, risk of avian predation is as high or even higher in these habitats, because sulphide-adapted fishes spend more time at the surface to perform aquatic surface respiration, exposing them to avian predators (Riesch *et al.*, 2010). It is therefore unlikely that potential differences in predation regimes between sulphidic and non-sulphidic habitats play a direct/major role for the reduction of brain size.

Still, sulphide-adapted fishes, which have smaller brains, show a weaker predator avoidance reaction when presented with piscine predator types compared to fish from non-sulphidic sites (Bierbach *et al.*, 2013). This is congruent with studies on guppies in which small-brained females had a lower survival rate than large-brained females when cohoused with a predator (Kotschal *et al.*, 2015). The higher survival rate of large-brained females was interpreted as being caused by a cognitive advantage, especially in the form of a more effective predator evasion behaviour (Kotschal *et al.*, 2015).

Smaller brains and reduced boldness in sulphide-adapted fishes

In guppies, brain size is further correlated with variation along the shy-bold behavioural axis: Fish with large brains are bolder in stressful situations, as indicated by a lower cortisol excretion (Kotschal *et al.*, 2014). Riesch *et al.* (2009) found reduced boldness in sulphide-adapted fishes (*P. mexicana* from Tac-S and the sulphidic cave; *P. sulphuraria*, Pich-S). Thus, smaller brains in sulphide-adapted fishes as found in our present study

and in Eifert *et al.* (2015) along with reduced boldness (Riesch *et al.*, 2009) are in full accordance with the findings by Kotschal *et al.* (2014). Interestingly, the degree of bold-shy differentiation among populations showed low heritability (Riesch *et al.*, 2009), which is in perfect congruence with the reported high phenotypic plasticity in brain area development (Eifert *et al.*, 2015).

Conclusions and Outlook

In all studied drainage systems, fishes from sulphidic and non-sulphidic habitats showed distinct differences in the size of total brains and brain regions that are very likely associated with differences in cognition and behaviour. Hence, we hypothesize that brain variation acts as a migration barrier (selection against migrants) between fishes from sulphidic and non-sulphidic habitats, and thus may contribute to reproductive isolation in each of the drainage systems. Even though the presence of H₂S is assumed to be one of the main drivers of ecological speciation in these drainage systems (reviewed by Riesch *et al.*, 2015) and brain divergence may be just interpreted as a direct or indirect response to the toxicant, increasing divergence in brain anatomy might further promote reproductive isolation. Previous studies in the *P. mexicana* species complex demonstrated strong natural and sexual selection against migrants from divergent habitat types even though there are no physical barriers preventing migration between different habitats within the drainages (Plath *et al.*, 2013). Selection against migrants is further brought about by behavioural differences (such as reduced aggression in Tac-S but not Pich-S males) hampering successful invasion (Bierbach *et al.*, 2012). Future studies are therefore warranted testing in how far brain (region) size variation in the *P. mexicana* complex is directly linked to these differences in behaviour and whether and how this variation may contribute to reproductive isolation. As sample sizes were partly small in our study, follow-up studies should analyse larger datasets that also include several non-sulphidic habitats in the upstream and downstream regions of each drainage system to further investigate the specific effects of H₂S on brain variation in *Poecilia* spp.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Descriptive statistics for eye diameter (ED), standard length (SL), brain mass, linear brain measurements and estimated brain (region) volume of male and female *Poecilia mexicana*, *P. sulphuraria* and *P. petenensis* from seven different populations.

Table S2. PCA using size-corrected measurements of length (L), width (W) and height (H) of the four brain regions (telencephalic lobes, optic tectum, hypothalamus, corpus cerebelli) as input variables.