

Compensatory behaviour in response to sulphide-induced hypoxia affects time budgets, feeding efficiency, and predation risk

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ABSTRACT

Background: In habitats containing toxic hydrogen sulphide, fish breathe at the water's surface to stay alive. This behaviour is called aquatic surface respiration.

Questions: What effects does this compensatory behaviour have? Does it constrain individuals' time budgets? Does it have a negative effect on foraging? Does it increase susceptibility to predators?

Organisms and locations: Atlantic mollies (*Poecilia mexicana*: Poeciliidae, Teleostei) and sulphur mollies (*P. sulphuraria*) inhabiting sulphidic and non-sulphidic habitats in Tabasco (Mexico).

Methods: We observed time budgets of fish in sulphidic and non-sulphidic habitats. We measured the amount of food eaten by fish in the various habitats. We exposed fish to a predator (giant water-bug, *Belostoma* sp.: Belostomatidae, Hemiptera) in mesocosms placed in sulphidic and non-sulphidic habitats.

Results: There was an inverse correlation between time spent performing aquatic surface respiration and time dedicated to foraging. Furthermore, fish in non-sulphidic habitats had more food in their guts than conspecifics from sulphidic habitats. Our predation experiments showed no overall difference in capture rates between sulphidic and non-sulphidic sites; however, males were disproportionately preyed upon.

Keywords: aquatic surface respiration, behavioural trade-off, *Belostoma*, cavefish, energy limitation, hydrogen sulphide, *Poecilia*, Poeciliidae.

INTRODUCTION

Physiochemical stressors affect the distributions of organisms on large (biogeographic) scales, and extreme conditions – such as can be found, for example, in polar regions, the deep sea, or deserts – provide the limits for the survival and reproduction of species or even larger taxonomic groups (MacArthur, 1972). Environmental conditions can be considered extreme if at least one physiochemical stressor lies outside the range normally experienced by a population, and the successful maintenance of homeostasis requires costly morphological, physiological, or behavioural adaptations absent in closely related taxa (Sibly and Calow, 1989; Townsend *et al.*, 2003). Such adaptations allow some organisms to thrive in places that are hostile for most others, giving rise to unique ecological communities [e.g. the arctic (Callaghan *et al.*, 2004); cold seeps (Kennicutt *et al.*, 1985); antarctic lakes (Laybourn-Parry and Pearce, 2007); deep sea hydrothermal vents (Van Dover, 2000)]. Extreme conditions may also occur on a much smaller spatial scale, nested within ‘benign’ habitats. Examples include hypoxic areas of lakes and swamps (Chapman *et al.*, 1995), highly acidic soils (Gough *et al.*, 2000), and areas with low water-holding potential within grasslands (Jose *et al.*, 1994). Such localized extreme habitats and their inhabitants are especially valuable for evolutionary ecology research because they allow for a powerful comparative approach examining the effects of physiochemical stressors on ecological and evolutionary processes without the confounding effects of major biogeographic patterns.

Extremophile fishes from sulphidic habitats

Localized extreme habitats occur in southern Mexican freshwaters in the form of springs and caves that are rich in naturally occurring hydrogen sulphide (H_2S) (Tobler and Plath, in press). Elevated H_2S concentrations in these springs are likely of volcanic origin (Rosales Lagarde *et al.*, 2006). Because of its lipid solubility, H_2S freely penetrates biological membranes and thus readily invades organisms (Reiffenstein *et al.*, 1992). Like cyanide, it is an inhibitor of the cytochrome *c* oxidase blocking the electron transport in aerobic respiration, thereby hampering the function of mitochondria and the production of ATP (Lovatt Evans, 1967; Nicholls, 1975; Petersen, 1977; National Research Council, 1979). Hence, H_2S is highly toxic for aerobic organisms even in micromolar amounts (Torrans and Clemens, 1982; Bagarinao, 1992; Grieshaber and Völkel, 1998). It is also highly reactive at room temperature and spontaneously oxidizes in aqueous solution (Cline and Richards, 1969; Chen and Morris, 1972). This reaction is biologically relevant because the presence of H_2S leads to and aggravates hypoxia in aquatic systems, resulting in a distinct inverse correlation of H_2S and oxygen concentrations (Bagarinao, 1992).

Due to their high toxicity [sulphide concentrations range from 15 to over 500 μM (Tobler *et al.*, 2006, 2008d)], the sulphidic springs are characterized by reduced species diversity compared with adjacent non-sulphidic habitats [similar to sulphide-rich hydrothermal vents and cold seeps (Kennicutt *et al.*, 1985; Price, 2002; Tsurumi, 2003)]. However, some of the springs are inhabited by small live-bearing fish of the family Poeciliidae [*Poecilia sulphuraria* and *Gambusia eurystoma* (Tobler *et al.*, 2008d); *Poecilia mexicana* (Tobler *et al.*, 2006)]. Compared with closely related species/populations from non-sulphidic habitats, sulphur spring residents appear to have specific adaptations that allow them to cope with the toxic and hypoxic conditions. In other organisms, physiological sulphide detoxification is achieved through oxidation of sulphide to less toxic compounds and subsequent excretion from the body [e.g. topminnows (Bagarinao, 1992); rats (Furne *et al.*, 2001); mudskippers (Ip *et al.*, 2004); humans (Picton *et al.*, 2002)]. Although the physiological pathways of sulphide detoxification in poeciliids remain to be studied, sulphide tolerance appears to have a strong heritable component, as laboratory-reared

animals from sulphidic habitats retain a higher tolerance to H₂S even when reared for several generations in a non-sulphidic environment (Peters *et al.*, 1973; reanalysed in Plath and Tobler, in press). Furthermore, H₂S spring residents have respiratory adaptations facilitating efficient oxygen acquisition, as oxygen available for respiration is not only limited due to the hypoxic conditions, but at the same time is required for the oxidative detoxification of H₂S. *Poecilia mexicana* from non-sulphidic and sulphidic habitats diverged primarily in head size and total gill filament length, whereby larger heads seem to allow for an increased gill area (Tobler *et al.*, 2008a).

Aquatic surface respiration as a compensatory behaviour

Despite the physiological and morphological adaptations, poeciliids in sulphidic springs are critically dependent on compensatory behaviour – that is, behaviour that is directly involved in coping with a stressor. When exposed to H₂S and hypoxia, they resort to aquatic surface respiration, where the fish exploit the more oxygen-rich air–water interface using their gills (Plath *et al.*, 2007c). Under experimental conditions, *P. mexicana* spent on average more than 60% of their time performing aquatic surface respiration when exposed to sulphidic water (Plath *et al.*, 2007c). In fact, the possibility to perform aquatic surface respiration is a strong predictor of short-term survival in fish exposed to sulphidic water (Plath *et al.*, 2007c). This compensatory behaviour has previously been reported in other fishes, including other poeciliids, when exposed to hypoxic (albeit non-sulphidic) conditions (Lewis, 1970; Kramer and Mehegan, 1981; Kramer and McClure, 1982; Kramer, 1983a; Winemiller, 1989; Timmerman and Chapman, 2004). In fish from temporarily hypoxic habitats, aquatic surface respiration imposes direct costs in terms of energy acquisition and use (Kramer, 1983b; Weber and Kramer, 1983) and an increased risk of aerial predation (Kramer *et al.*, 1983). In this study, we quantified potential costs of this compensatory behaviour in sulphide spring residents of the genus *Poecilia* using field observations and experiments.

Compensatory behaviour and energy acquisition

In the first part of our study, we wished to determine whether and how aquatic surface respiration affects foraging. Fish of the genus *Poecilia* are benthic feeders. In addition to detritus, they consume algae in non-sulphidic habitats and aquatic insects in sulphidic habitats (Tobler, 2008). Performing aquatic surface respiration should therefore give rise to trade-offs in the allocation of time towards foraging, as fish can only be either at the water surface for respiration or at the bottom for feeding. Such a trade-off between compensatory behaviour and energy acquisition could be a potential explanation for the eminently low body condition of fish living in sulphidic habitats (Tobler, 2008; Tobler *et al.*, 2006) despite the supposedly high availability of resources in such habitats through chemoautotrophic bacterial primary production (Langecker *et al.*, 1996). We used two approaches to study potential trade-offs between aquatic surface respiration and foraging: (1) we assessed the allocation of time to different behaviours (time budgets) by direct observation of *Poecilia* spp. in several sulphidic and non-sulphidic surface habitats; and (2) we compared the amount of food items in the guts of individuals from sulphidic and non-sulphidic surface and cave habitats as an indicator of foraging efficiency in natural populations.

Compensatory behaviour and predation risk

In the second part of the study, we tested the hypothesis that reliance on aquatic surface respiration renders fish in sulphidic habitats more susceptible to predation. Since fish relying on this behaviour spend extended periods of time at the water surface, they are expected to be more susceptible, especially to predators attacking from above the water surface (Kramer *et al.*, 1983), although not necessarily aquatic predators (Poulin *et al.*, 1987). We examined predation by one of the few predators occurring in sulphidic streams in our study system: a giant water-bug of the genus *Belostoma* (see Gordon and Rosen, 1962). Giant water-bugs are sit-and-wait predators that catch bypassing prey items with their raptorial forelegs while sitting at the edge of the water holding their abdomen in the air to breath (Menke, 1979). These hemipterans have previously been documented to prey upon *P. mexicana* in the sulphidic cave investigated here (Plath *et al.*, 2003; Tobler, 2009; Tobler *et al.*, 2007b). Males appear to be more susceptible to *Belostoma* predation, as they allocate more time to aquatic surface respiration than females (Tobler *et al.*, 2008b). In this study, we experimentally exposed male and female *P. mexicana* to predation by *Belostoma* under different H₂S concentrations to examine differences in susceptibility to predation under different environmental conditions. We predicted that (1) overall, predation would be stronger at higher H₂S concentrations, and (2) males would be disproportionately more prone to predation.

Our current study documents direct ecological consequences associated with compensatory behaviour necessary to cope with extreme environmental conditions in sulphidic habitats. Knowledge about these ecological consequences allows for a better understanding of the complex differences in selective regimes between sulphidic and adjacent non-sulphidic habitats that may account for trait divergence – ranging from behaviour to morphology – exhibited by fish from the different habitat types (Parzefall, 2001; Plath and Tobler, in press).

MATERIALS AND METHODS

Study systems

We studied costs of aquatic surface respiration in two sulphidic systems, the Cueva del Azufre system and in the Baños del Azufre. All sites are located near the city of Teapa, Tabasco, Mexico (Table 1). The Cueva del Azufre system includes four different habitat types that are characterized by the presence or absence of H₂S and light: a sulphidic cave (Cueva del Azufre), a non-sulphidic cave (Luna Azufre), sulphidic surface habitats (El Azufre; consisting of discharge from the Cueva del Azufre as well as independent sulphidic springs located at the surface), and several non-sulphidic surface habitats. Details on the abiotic and biotic conditions in the different habitat types have been published elsewhere (Tobler *et al.*, 2006, 2008a, 2008c). *Poecilia mexicana* are abundant in the extreme habitats, and there are several lines of evidence for local adaptation – that is, morphologically and genetically distinct lineages with discrete life histories inhabiting different habitat types (Tobler *et al.*, 2008a; Riesch *et al.*, 2009). Indeed, gene flow among habitats differing in environmental conditions is low (Plath *et al.*, 2007a; Tobler *et al.*, 2008a).

The Baños del Azufre are an independent system of sulphidic springs that harbour another population of *Poecilia* living in highly sulphidic water (Tobler *et al.*, 2008d). This population is morphologically highly divergent and has been described as a distinct species, *Poecilia sulphuraria* (Alvarez del Villar, 1948).

Table 1. List of collection sites (abbreviations used throughout the article appear in parentheses after the name), their location (latitude, longitude), and the number of individuals examined/replicates performed at each site in the different parts of the study

Site	Location	Time budgets	Foraging efficiency	<i>Belostoma</i> predation
Non-sulphidic surface habitats				
Arroyo Bonita (AB)	17.42706, -92.75194	50		
Arroyo Cristal (AC)	17.45063, -92.76369	51		
Arroyo Tacubaya (AA)	17.45355, -92.78449	20	28	
Río Oxolotan (RO)	17.44444, -92.76293			30
Sulphidic surface habitats				
El Azufre I (EAI)	17.44225, -92.77447	54	33	
El Azufre II (EAI)	17.43852, -92.77475	51	41	
Baños del Azufre (PS)	17.55227, -92.99897	54		
Non-sulphidic cave habitat				
Cueva Luna Azufre (LA)	17.44171, -92.77312 ^a		41	
Sulphidic cave habitat				
Cueva del Azufre, chamber V (V)	17.44234, -92.77542 ^a		38	28
Cueva del Azufre, chamber X (X)			40	
Cueva del Azufre, chamber XIII (XIII) ^b				28

Note: With the exception of the Baños del Azufre population (*P. sulphuraria*), all investigated *Poecilia* populations were *P. mexicana*.

^a Location of the cave entrance.

^b Although located within a sulphidic cave, chamber XIII is non-sulphidic and exhibits high oxygen concentrations, since this hind-most chamber is located upstream of the uppermost sulphidic spring in chamber XI.

Time budgets

Time budgets were assessed at six sites (Table 1). Observers sat quietly next to a stream pool. We used a scan sampling approach (Martin and Bateson, 1986) to assess the duration of feeding behaviour and aquatic surface respiration, while swimming (e.g. moving between feeding patches), sexual, and aggressive behaviours were recorded as 'other behaviours'. The minimum observation time of individual fish was defined *a priori* as 60 s, and observations lasted for a maximum of 600 s (mean \pm s.d. observation time: 107 ± 61 s).

We tested for a correlation between the average proportion of time spent feeding and performing aquatic surface respiration using a non-parametric Kendall correlation on population means. We also tested for a correlation between time spent feeding and performing aquatic surface respiration within populations that exhibited compensatory behaviour using Pearson correlations.

Foraging efficiency

We used the mass of food items in the gut as a proxy for foraging efficiency. Fish were collected at two sites in non-sulphidic surface habitats, two sites in sulphidic surface

habitats, one cave chamber in the non-sulphidic Luna Azufre, and two chambers in the sulphidic Cueva del Azufre (Table 1). All specimens were euthanized using MS222 and preserved in 10% formaldehyde solution immediately after capture. Fish were then dissected for a gut content analysis (see Tobler, 2008). We weighed the mass of the gut content as well as the total mass of each fish to the closest 0.01 g. We used analysis of covariance (ANCOVA: $R^2 = 0.78$) to test for the effects of body weight (covariate) and the presence or absence of H_2S and light (independent variables). The interaction effects were not significant ($F \leq 0.69$, $P \geq 0.41$), suggesting homogeneity of slopes, thus only main effects were analysed. Effect sizes were approximated using partial eta-squared (η_p^2).

Predation risk

Fish and giant water-bugs for the predation risk experiment were collected in cave chamber V of the Cueva del Azufre. The experiments were performed in 2-litre PET bottles that were perforated with around 30 small (<5 mm) holes to allow for air and water exchange (Tobler *et al.*, 2007b, 2008b). A male and a female *P. mexicana*, matched for size (standard length, mean \pm s.d.: males, 28.0 ± 4.0 mm; females, 28.0 ± 4.4 mm; paired *t*-test: $t_{55} = 0.00$, $P = 1.00$), were introduced into a bottle together with a water-bug (length from the tip of the head to the end of the abdomen, mean \pm s.d.: 16.9 ± 4.2 mm). Bottles were then placed in a shallow area within the cave and fixed in place with rocks. We conducted 56 replicates, 28 of which were placed in the sulphidic cave chamber V and 28 in the non-sulphidic chamber XIII, which lies upstream of the uppermost sulphidic spring [see Tobler *et al.* (2006) for water chemistry data]. Bottles were only partially submerged to allow the water-bugs to breathe and the fish to perform aquatic surface respiration (Tobler *et al.*, 2007b, 2008b). After 24 h, we checked the replicates for the presence of puncture wounds in fish. All fish and water-bugs were released at the original collection site following the experiment. We tested for differential predation between habitats and sexes using a logistic regression with binomial errors with the following model: attack (successful/unsuccessful) = block (mesocosm) + habitat + sex + habitat*sex.

RESULTS

Time budgets

Fish from non-sulphidic habitats spent more than half of their time feeding and did not exhibit any aquatic surface respiration (see Fig. 1A for descriptive statistics on an individual basis, Fig. 1B for population averages). In contrast, *Poecilia* spp. from sulphidic habitats spent between 8 and 84% of their time performing aquatic surface respiration, but spent only between 3 and 30% of their time feeding. Across populations, time spent feeding was negatively correlated with the average time spent performing aquatic surface respiration (Kendall's $\tau = -0.89$, $P = 0.02$, $n = 6$). Within populations, aquatic surface respiration was negatively correlated with time spent feeding in the EAI population ($r = -0.37$, $P < 0.01$, $n = 54$) and the PS population ($r = -0.32$, $P = 0.02$, $n = 54$), but not the EAI population ($r = -0.06$, $P = 0.70$, $n = 52$).

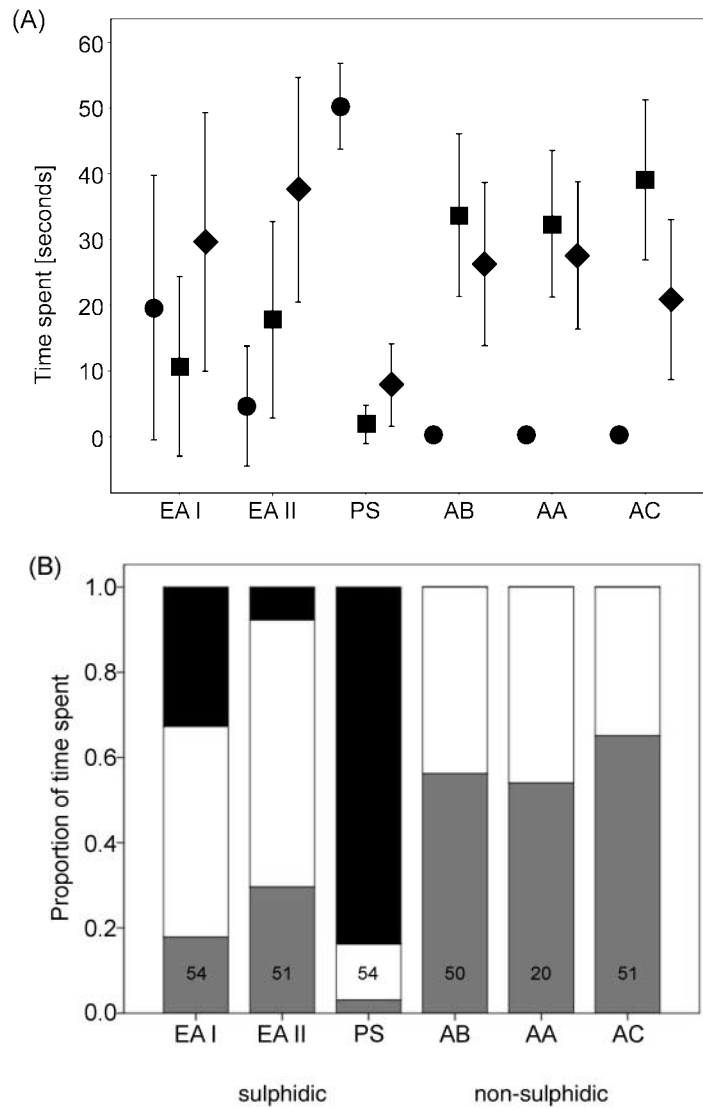


Fig. 1. (A) Mean (\pm s.e.) number of seconds individuals spend performing aquatic surface respiration (●), feeding (■), and exhibiting other behaviours (◆) per minute. (B) Population averages of the same measurements (aquatic surface respiration: black; feeding: grey; other behaviours: white). Numbers in the bars represent the number of individuals observed. See Table 1 for locations.

Foraging efficiency

The amount of food in an individual's gut was positively correlated with body mass (ANCOVA: $F_{1,247} = 644.2$, $P < 0.01$, $\eta_p^2 = 0.72$). Fish from sulphidic habitats had significantly less food in their guts than fish from non-sulphidic habitats ($F_{1,247} = 84.9$, $P < 0.01$, $\eta_p^2 = 0.26$), while there was no significant difference between fish from cave and surface habitats ($F_{1,247} = 1.95$, $P = 0.16$, $\eta_p^2 = 0.01$) (Fig. 2).

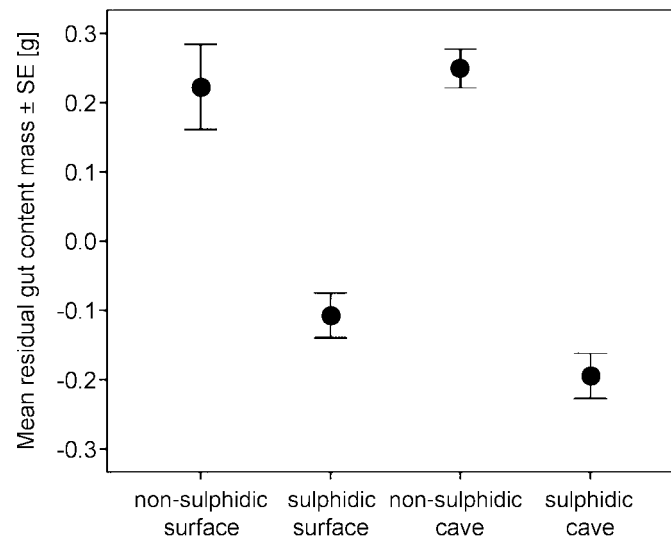


Fig. 2. Mean (\pm s.e.) residual gut content mass in different populations of *P. mexicana* in the Cueva del Azufre system. We calculated residuals using a linear regression between body mass and gut content mass to control for differences in size.

Table 2. Logistic regression on *Belostoma* attacks (successful/unsuccessful) on *P. mexicana*

	<i>B</i>	s.e.	Wald	d.f.	<i>P</i>
Block (mesocosm)	-0.02	0.01	0.03	1	1.00
Habitat	0.10	0.12	0.67	1	0.41
Sex	3.50	1.41	6.17	1	0.01
Habitat \times sex	-0.22	0.14	2.57	1	0.11

Note: -2 Log likelihood = 107.5; Cox & Snell R^2 = 0.12; Nagelkerke R^2 = 0.18.

Predation risk

The overall capture rate was slightly higher at the sulphidic site [predation had occurred in 15 of 28 replicates (54%) at the sulphidic site versus 10 out of 28 (36%) at the non-sulphidic site; Fig. 3], but the difference was not statistically significant (Table 2). Sex was the only significant predictor of susceptibility to predator attacks in our experiment (Table 2).

DISCUSSION

Sulphidic springs challenge their inhabitants with acutely toxic concentrations of H_2S and low concentrations of oxygen, which is needed for respiration as well as the detoxification of H_2S . Experimental studies suggested that aquatic surface respiration, during which fish exploit the oxygen-rich air–water interface, allows fish to cope with the adverse environmental conditions (Plath *et al.*, 2007c). Here, we confirmed this finding for several populations of *Poecilia* spp. in their natural habitats. Both *P. mexicana* and *P. sulphuraria* inhabiting

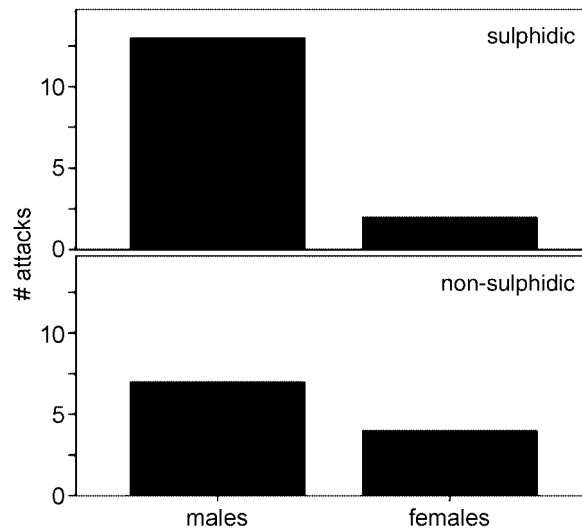


Fig. 3. Number of male and female *P. mexicana* preyed upon by *Belostoma* in sulphidic and non-sulphidic water. Males were more susceptible to predator attacks, but there was no difference between sulphidic and non-sulphidic environments (Table 2).

sulphidic springs dedicate a substantial proportion of their time to performing aquatic surface respiration, while fish in non-sulphidic habitats do not exhibit this behaviour at all.

Aquatic surface respiration, foraging, and energy limitation

In natural habitats, there appears to be a trade-off in the amount of time an individual fish can allocate to either compensatory behaviour or foraging. Across populations, the time fish spent performing aquatic surface respiration was negatively correlated with time spent foraging. Such a negative correlation could also be found within populations from sulphidic habitats with the exception of the EAII population. Compared with the other two sulphidic populations, this particular population exhibited aquatic surface respiration least frequently. The low frequency of aquatic surface respiration and the lack of a trade-off between aquatic surface respiration and foraging could be related to the proximity of the EAII site to non-sulphidic microhabitats. While fish at the EAI and PS sites have no opportunity to avoid sulphidic water, *P. mexicana* at EAII can move upstream into non-sulphidic water, as there is a steep drop in H_2S concentration above the sulphidic springs (M. Tobler, unpublished data). Like other fish in temporarily hypoxic habitats (Van Den Thillart and Verbeek, 1991; Chew and Ip, 1992), *P. mexicana* at this site may cope with low oxygen availability by switching to anaerobic metabolism and later repay the accumulated oxygen debt by moving into more favourable microhabitats (i.e. fish in this population may actually have an alternative to aquatic surface respiration for assuring efficient oxygen acquisition). Foraging this way (i.e. spending periods of foraging followed by interruptions while retreating in normoxic waters) may still impose constraints on the time budget, and searching for prey items during the limited time might make fish more conspicuous and susceptible to predator attacks.

A second line of evidence unravelled a constraining effect of aquatic surface respiration on foraging behaviour. Fish from sulphidic habitats were found to have significantly less food in their intestinal tract than fish from non-sulphidic habitats. This is true not only for surface habitats, but also for cave habitats, suggesting that the presence of H₂S has a stronger effect on individuals' foraging efficiency than the absence of light. *Poecilia mexicana* are benthic feeders, and populations living in sulphidic habitats went through a pronounced ecological shift, changing their diet from predominantly detritus and algae in normal water to detritus and aquatic invertebrates (Tobler, 2008). Given the constraint imposed by aquatic surface respiration, it could also be expected that fish in sulphidic habitats would forage more at the water's surface and, for example, consume more terrestrial insects. However, the composition of the diet (Tobler, 2008) as well as behavioural observations in the field (this study) suggest that fish in sulphidic habitats remain benthic feeders.

The trade-off between compensatory behaviour and foraging may provide a powerful proximate mechanism to explain the poor body condition of fish in sulphidic habitats as well as the evolution of traits that appear to be adaptations to energy limitation. Sulphur spring residents in the Cueva del Azufre system have an eminently low body condition (Tobler *et al.*, 2006; Tobler, 2008). Bodily energy reserves appear to be so low that energy availability critically affects the survival of the fish in their natural habitat even over very short periods of time (24 h), and fish that were fed with energy-rich food were more likely to survive under semi-natural conditions (Plath *et al.*, 2007c). This contrasts with the finding that energy resources (detritus, chironomids, and bat guano) appear to be abundant, since productivity in sulphidic habitats is thought to be high due to chemoautotrophic primary production (Langecker *et al.*, 1996). Furthermore, inter-specific resource competition is low (Tobler *et al.*, 2006).

We found that energy sources (though abundant) may not be readily accessible for the fish because of constraints imposed by the large proportion of compensatory behaviour in the activity budget. But even if fish were able to maintain high foraging rates, they may not have the physiological capacity to increase their body mass, if their aerobic scope is limited or if they are unable to acquire sufficient oxygen to fuel the aerobic processes associated with tissue production. Interestingly, sulphidic spring residents in the Cueva del Azufre system have evolved several traits that appear to be adaptations to energy scarcity such as reduced shoaling behaviour (Plath and Schlupp, 2008), reduced male sexual activity (Plath *et al.*, 2003, 2007b), and shifts in life-history strategies (Riesch *et al.*, 2009).

Sulphide and *Belostoma* predation

Aquatic surface respiration has previously been shown to increase the risk of predation by aerial and hypoxia-tolerant predators (Kramer *et al.*, 1983; Domenici *et al.*, 2007). Hence, we expected predation by giant water-bugs, which capture fish from the water surface, to be higher under high H₂S, but this was not the case. Overall predation rates did not differ significantly between non-sulphidic and sulphidic sites, but this could be caused by the small size of the mesocosms or the duration of the experiment. However, males were more susceptible to predation habitats than females. Male-biased predation has previously been reported in this system (Tobler *et al.*, 2008b). It remains to be studied how the performance of aquatic surface respiration affects susceptibility to predators in surface habitats where piscivorous birds are common (Tobler *et al.*, 2007a).

In summary, compensatory behaviour in the form of aquatic surface respiration appears

to have a strong effect on time budgets and foraging efficiency in *Poecilia* living in sulphidic habitats. We propose that trade-offs in time allocation imposed by compensatory behaviour may be a proximate cause for the energy deprivation of sulphide spring residents, giving rise to the evolution of traits that minimize energy expenditure.

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