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 $\mu$M (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 \( \text{H}_2\text{S} \) even when reared for several generations in a non-sulphidic environment (Peters et al., 1973; reanalysed in Plath and Tobler, in press). Furthermore, \( \text{H}_2\text{S} \) 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 \( \text{H}_2\text{S} \). *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 \( \text{H}_2\text{S} \) 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 chemosynthetic 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 H$_2$S concentrations to examine differences in susceptibility to predation under different environmental conditions. We predicted that (1) overall, predation would be stronger at higher H$_2$S 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 H$_2$S 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).
Time budgets

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 ± 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$_2$S 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 ($\eta^2_p$).

**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 ± 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 ± 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 EAII population ($r = −0.06$, $P = 0.70$, $n = 52$).
Foraging efficiency

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

Fig. 1. (A) Mean (± 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.
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$_2$S and low concentrations of oxygen, which is needed for respiration as well as the detoxification of H$_2$S. 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
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 EAI 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$_2$S 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 H$_2$S 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 H$_2$S, 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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