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Survival in an extreme habitat: the roles of behaviour and energy limitation

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Abstract Extreme habitats challenge animals with highly adverse conditions, like extreme temperatures or toxic substances. In this paper, we report of a fish (*Poecilia mexicana*) inhabiting a limestone cave in Mexico. Several springs inside the cave are rich in toxic H_2S. We demonstrate that a behavioural adaptation, aquatic surface respiration (ASR), allows for the survival of *P. mexicana* in this extreme, sulphidic habitat. Without the possibility to perform ASR, the survival rate of *P. mexicana* was low even at comparatively low H_2S concentrations. Further-more, we show that food limitation affects the survival of *P. mexicana* pointing to energetically costly physiological adaptations to detoxify H_2S.

Keywords Aquatic surface respiration · Cave fish · Extremophile · Hypoxia · Hydrogen sulphide

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Introduction

Extreme habitats are characterised by harsh conditions and require, of any organism tolerating these, costly adaptations absent in related species (Townsend et al. 2003). For example, some extreme habitats are characterised by high concentrations of hydrogen sulphide (H_2S), which competes with oxygen in the respiratory chain and is highly toxic for animals even in micro-molar amounts (Torrans and Clemens 1982; Bagarinao 1992; Grieshaber and Völkel 1998). Almost all known metazoans from sulphidic habitats are invertebrates, which cope with naturally occurring H_2S by (1) avoiding micro-habitats with high sulphide concentrations, (2) switching to anaerobic metabolism, (3) excluding sulphide from sensitive tissues or (4) oxidising sulphide to more benign forms (Grieshaber and Völkel 1998; McMullin et al. 2000 for reviews).

Despite the toxicity of H_2S, sulphidic habitats can harbour thriving ecosystems, e.g. in sulphidic caves (Sarbu et al. 1996) or near deep-sea hydrothermal vents (Van Dover 2000). Such sulphidic habitats are characterised by high productivity relative to similar habitats. While deep-sea or cave ecosystems rely on nutrient influx from the euphotic zone or surface habitats, respectively (Poulson and Lavoie 2000), sulphidic habitats can support ecosystems based on chemoautotrophic microbial primary production (Jorgensen 1984; Sarbu et al. 1996).

One of the few vertebrates known from a permanently sulphidic habitat is a cave form of a poeciliid fish, the cave molly (*Poecilia mexicana*), from the southern Mexican Cueva del Azufre (Gordon and Rosen 1962). Several H$_2$S-rich springs feed a creek that runs through the cave. Measurements of H$_2$S in the water read up to 300 μM (Tobler et al. 2006), which is acutely toxic for most animals (e.g. Smith et al. 1976; Bagarinao 1992). Oxygen concentrations are inversely correlated with H$_2$S and range between 3.8 and 32.0% saturation equalling extreme hypoxia (Tobler et al. 2006).

How the cave molly copes with its toxic environment is currently unknown. In laboratory studies, air breathing has been suggested to mediate the survival in temporarily sulphidic environments in some species of fish (Bagarinao and Vetter 1989; Brauner et al. 1995; Affonso and Rantin 2005). A behavioural equivalent to air breathing is aquatic surface respiration (ASR), during which the air–water interface is exploited. Many fishes, especially poeciliids, employ ASR when encountering hypoxic conditions (Kramer and McClure 1982).

In the present study, we experimentally investigated whether cave mollies rely on ASR during exposure to sulphidic water and whether this behaviour mediates their survival in the natural habitat. ASR is physiologically costly and fundamentally alters the time budget of individuals (Kramer 1983). If ASR was a crucial mechanism for cave mollies to cope with H$_2$S, we would also expect food availability to affect their survival. For this, we used two independent approaches: We measured survival rates of fish with or without access to the water surface and with or without an energy-rich food source. In addition, we determined the body condition for several populations of mollies.

Materials and methods

Model system

Field work was conducted in August 2004 in the Cueva del Azufre, near Tapijulapa (Tabasco, Mexico; 17°26.5′N, 92°46.5′W). The cave is divided into 13 chambers and a creek, fed by springs in the rear chambers, drains the cave (Gordon and Rosen 1962). The creek (El Azufre) eventually leaves the cave and runs for several kilometres, before it drains into the next larger river, the Río Oxolotan (Tobler et al. 2006). Most sulphidic springs are located in cave chamber X. On its way from chamber X to chamber I, the water flows over a number of riffles at which H$_2$S is gradually oxygenated in the water. This leads to a pattern of high H$_2$S deep in the cave, less H$_2$S towards the cave exit and even less H$_2$S in the El Azufre. However, the lower

values measured near the cave exit are still considered toxic for most animals (Tobler et al. 2006).

The general ecology of the cave is not well understood, but it seems that the food web is based on energy input through bat guano and primary production through sulphide oxidising bacteria. These bacteria are abundant in the cave and coat every aquatic surface. The cave molly is a genetically differentiated population of a widespread surface-living species, the Atlantic molly (*P. mexicana*, Poeciliidae; Gordon and Rosen 1962; Plath et al. 2007). Cave mollies differ from surface Atlantic mollies in a set of characters including reduced but still functional eyes and reduced pigmentation (reviewed in Parzefall 2001). It has been suggested that cave mollies feed on detritus, bat guano, bacteria and small invertebrates living in the cave (Langecker et al. 1996).

Population comparison of condition factors

In previous experiments, we demonstrated that cave molly males in nature have the same relative distension of their abdomen as males from a laboratory stock after 1 week of starvation (Plath et al. 2005). This pointed towards a role for energy limitation in this system, and thus we compared the condition factor (CF) between *P. mexicana* (*n*=610) from cave chambers V, XI and XIII and mollies from two adjacent surface habitats (the sulphidic El Azufre and a nearby clear-water creek, Arroyo Cristal). We determined the standard length (SL [mm] using a millimetre grid) and the weight (*M* [g] using a Pesola scale) of individual fish and calculated the CF as $1,000 \times M/\mathrm{SL}^3$. Measuring accuracy with the Pesola scale was probably imperfect, but a more accurate scale was not available in the field. All measured fish were released again on site. Data were analysed using 'population' as between factor and 'sex' as within factor for a two-way analysis of variance (ANOVA). Because the interaction term was not significant ($F_{4,\ 599}=0.16$, $P=0.96$), only the main effects were analysed.

Influence of surface access and food on survival

In a field experiment, we investigated the importance of access to the water surface and food availability for the survival of cave mollies in the natural habitat. 115 adult *P. mexicana* were caught in chamber V. Standard length, weight and sex of each individual were determined and the CF was calculated. Each fish was transferred into a perforated plastic box (9.5×9.5×6 cm), which contained either a small amount of detritus (the natural, ubiquitous food source), no food or one tablet of protein-rich fish food (Tetra® TabiMin). The perforation allowed water to flow through the containers, but detritus and food were retained. Boxes were positioned inside cave chamber V in shallow

areas with slow water current and were fixed in place with stones. Mollies occurred in this particular microhabitat, and fish were observed swimming between the experimental boxes. We used a conservative approach and conducted the experiment in chamber V, which is downstream from chamber X, and has much lower values for H_2S (maximum 32 μM; Tobler et al. 2006). One half of the boxes containing fish were fully submerged, the other half remained half-filled with air, allowing the fish to exploit the air–water interface and perform ASR.

Data were analysed using a logistic regression. In the case of the food treatments, the detritus treatment was used as reference level because cave mollies may naturally feed on detritus (Langecker et al. 1996). For the factor 'sex,' male sex was arbitrarily used as the reference level.

This experiment was originally designed to run for a long period of time using growth rate as the dependent variable. When we checked the setup for the first time after 24 h, we discovered that many fish had died in the treatments that did not allow access to the air–water interface, so we immediately terminated the experiment and released the remaining fish. Monitoring the experiment overnight would have been very difficult because of the high concentration of H_2S in the air. In an unrelated laboratory study, Brauner et al. (1995) reported *Hoplosternum littorale* surviving 200 μM H_2S when forced under water. In our study, we exposed fish to maximum 32 μM H_2S at the site of the experiment. Furthermore, we had often observed fish swimming underwater, not conducting ASR; hence, we were highly surprised by the high mortality.

Behavioural responses: aquatic surface respiration

The result of the surface-access experiment prompted us to quantify ASR when cave mollies are exposed to sulphidic water. The experiment was conducted just outside of the cave between 12:00 and 16:00 hours in a shaded location. Thirty-five mollies were randomly collected in cave chamber V using dip nets. Each fish was subjected to two sequential treatments: (1) water from a spring in cave chamber X, which contained 309 μM of H_2S (Tobler et al. 2006), and (2) water from the same spring, which was detoxified by 1 h of intensive aeration. This treatment can successfully reduce H_2S to 0 μM (Tobler et al., unpublished data). A single fish was introduced into a clear plastic bottle filled with 300 ml of water. We gave the fish 5 min for habituation, and then the time until first surface contact (latency) and the time spent at the surface performing ASR were measured for 5 min. Afterwards, the fish was transferred into another bottle with water from the opposite treatment, and the measurement was repeated. The order of the treatments was balanced. We used 'sex' as the between factor, 'order' of the treatments as the within factor and

'ASR' (and 'latency time', respectively) under the two water treatments as the dependent variable for a repeated-measures ANOVA.

Results

Population comparison of condition factors

We found significant differences in CFs among populations (ANOVA: $F_{4, 599}=48.39$, $P<0.0001$). Surface fish from a separate, sulphide-free habitat had higher CFs (mean±SD: $0.032±0.011$ mg mm^{-3}) than the cave fish (chamber V= $0.020±0.010$; chamber XI=$0.020±0.033$; chamber XIII= $0.018±0.043$) and fish from the sulphidic El Azufre ($0.023±0.049$), which in turn had higher CFs than fish from the cave (Tukey test, $P<0.05$ in all cases). No sex-specific differences were found ($F_{1, 599}=1.60$, $P=0.21$).

Influence of surface access and food on survival

The results from our unintentional experiment of mortality rates relative to access to the water surface and food were analysed using a logistic regression ($R^2=0.34$, log likelihood=−51.74, intercept log likelihood=−78.45). They indicated that mortality of cave mollies after 24 h was significantly lower when the fish had access to the water surface (Fig. 1; Table 1). Furthermore, supply with food tablets lead to significantly higher survival of the fish (Fig. 1; Table 1). Sex also had a significant effect, and males performed worse. The CF or body size had no influence on the mollies' survival in this experiment (Table 1). We conducted an additional, exploratory analysis using residuals to look for interactions between the food treatment and ASR and found no significant results (analysis not shown).
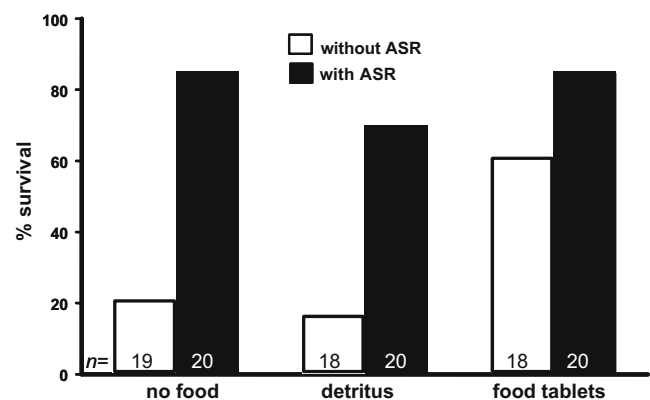

Fig. 1 Survival-rate of cave mollies (in percent) when housed under semi-natural conditions with (*black*) or without access to the water surface (*white*) for 24 h. The fish were either fed no food (*left*), detritus (*middle*) or food tablets (*right*)

Table 1 Survival with or without possibility for ASR

	c	χ^2	P
Standard length	0.026	0.091	0.76
Condition factor	0.006	2.19	0.14
Feeding −	−0.78	1.69	0.19
Feeding +	+1.96	8.75	0.0031
Access to surface	2.93	23.43	<0.0001
Sex	2.00	6.18	0.0013

Cave mollies were fed detritus (reference level), food tablets (+) or no food (−). The fish either had an opportunity for ASR (access to the surface) or not. Logistic regression on survival after 24 h.

Behavioural responses: aquatic surface respiration

Cave mollies spent significantly more time showing ASR in H₂S-rich water than in oxygenated water in our field experiment (Table 2). Males spent significantly more time at the surface than females both in H₂S-rich water (mean± SD, males=85.8±33.7%, females=63.1±44.8%) and in oxygenated water (males=26.7±31.8%, females=5.4± 14.4%). Likewise, water treatment and sex strongly affected the latency time (Table 2) to the water surface (H₂S-rich water=37.5±102.5 s [males], 69.4±123.9 s [females]; oxygenated water=97.3±130.5 s [males], 223.7±116.9 s [females]).

Discussion

Unlike most other fishes, cave mollies have to cope with sustained and highly toxic concentrations of hydrogen sulphide, coupled with low concentrations of oxygen, in their natural habitat (Tobler et al. 2006). One way in which cave mollies deal with this harmful situation is by

Table 2 Repeated-measures ANOVA on time spent at the surface (ASR) and latency time until first surface contact in water from a sulphidic spring and oxygenated water from the same locality

	$F_{1,\ 34}$	P
ASR		
Order	2.03	0.16
Sex	5.86	0.021
Water treatment	60.82	<0.0001
Latency time		
Order	0.23	0.63
Sex	8.80	0.0057
Water treatment	11.44	0.0019

Because the interaction-terms were not significant ($P>0.1$), only the main effects were analysed.

performing ASR, a behaviour that allows them to exploit the one layer in the water column with the highest relative oxygen—and the lowest relative sulphide-concentration. At the air–water interface, diffusion is responsible for keeping the oxygen concentration high, which in turn oxidizes and thus detoxifies H₂S. ASR has been documented in poeciliids (e.g. Kramer and Mehegan 1981; Timmerman and Chapman 2004a) and other fishes (Kramer and McClure 1982; Chapman et al. 1995) and is a plastic behaviour; individuals can adjust their trips to the surface to reflect increasing oxygen consumption and/or decreasing oxygen content in their environment (Timmerman and Chapman 2003, 2004a, b; this study). ASR is especially pronounced in cave mollies, which spent most of their time (on average 73.4%) breathing at the surface when exposed to high H₂S concentrations. Compared to closely related sailfin mollies (*P. latipinna*), cave mollies showed considerably more ASR under chronic hypoxia (1 mg/l O₂; Timmerman and Chapman 2003). In fact, sailfin mollies that were well adapted to hypoxia still spent less than 20% of their time budget performing ASR (Timmerman and Chapman 2004a).

That the ability to perform ASR is critical to cave mollies' survival in their natural habitat is evidenced by the significantly increased mortality when fish did not have access to the water surface, even at comparatively low levels of H₂S (32 μM). Similar findings have been documented in other fishes (Bagarinao 1992; Brauner et al. 1995; Affonso and Rantin 2005).

Overall, the ability of cave mollies to use ASR may be a plesiomorphic trait that enabled fish to successfully colonise the Cueva del Azufre. *P. mexicana* from non-sulphidic habitats respond with very low levels of ASR and a rapidly loose motion control when exposed to H₂S-rich water (Plath et al., unpublished data), which implies that that the colonizers and their descendants have experienced strong selection for increasing rates of ASR. Importantly, neutral molecular data indicate that there is no persistent gene flow from the outside into the cave, which would weaken the effects of such selection within the cave environment (Plath et al. 2007).

This is not, however, the whole story: Our findings indicate that cave mollies may also be energy limited. Body condition is significantly worse in these fish than in their surface-dwelling relatives, so, not surprisingly, supplementary feeding with a high protein food increased survival rates when the fish were placed under stressful conditions. The hypothesis that cave mollies are energy limited is supported by the observation that they show a heritable reduction of energy-consuming behaviours involved in aggressive (Parzefall 2001) and sexual interactions (Plath et al. 2003). Male cave mollies initially appear to be the most energy limited by their living conditions. Compared

to females, they exhibited a higher mortality under stressful conditions and performed longer ASR trips. It is possible that the cost of living under these extreme conditions has been manifested differently in females. Overall however, the results of this and other studies indicate that cave mollies are energetically stressed, which is odd given that the Cueva del Azufre has been characterised as an energy-rich system based on sulphide-oxidising bacteria (Langecker et al. 1996). This paradox may be explained in two, not necessarily mutually exclusive, ways: (1) although energy rich, the cave ecosystem may lack particular nutrients that are essential to the fishes' health. This possibility cannot be examined further until we have delineated the composition of all available foods in the cave, and (2) some mechanism essential to survival in the H_2S-rich habitat may be depleting an individual's energy budget. ASR qualifies as such a mechanism. It is physiologically costly (Kramer 1983), and its performance decreases the amount of time available for other behaviours such as foraging (Weber and Kramer 1983; Chapman and Chapman 1993). Either of the preceding factors or their combination could cause cave mollies to exhibit a poor body condition in a purportedly energy-rich habitat.

The interaction between condition and ASR appears to be a complicated one. Importantly, fish denied ASR showed very high mortality levels except in the group that had been supplemented with food tablets (Fig. 1). This implies that, in addition to ASR, there might be a mechanism responsive to the intake of nutrient/energy-rich food contributing to these fishes' survival in H_2S-rich waters.

It is not clear yet how cave mollies cope with the potential long-term toxic effects of sulphide even at low concentrations. Other fishes have evolved physiological adaptations to cope with H_2S in their environment; for example, they are able to enzymatically oxidise sulphide and subsequently excrete it from the body (reviewed in Bagarinao 1992). While behaviour may be one key to the survival of cave mollies in their natural habitat, morphological and physiological adaptations to H_2S clearly warrant further investigations.

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