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Reduced starvation resistance and increased metabolic rates in an unusual cave organism: the cave molly (*Poecilia mexicana*, Poeciliidae)

Geringere Hungerresistenz und gesteigerte Stoffwechselraten bei einem ungewöhnlichen Höhlentier, dem Höhlenmolly (*Poecilia mexicana*, Poeciliidae)

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Summary: Caves are traditionally thought to be resource-limited environments and consequently most cave-dwelling organisms examined have evolved increased starvation resistance and reduced metabolism (hypometabolism). Here, both aspects are investigated in the neotropical livebearing fish *Poecilia mexicana* from a toxic (H_2S-rich), but highly productive cave and several populations from H_2S-free surface habitats. *P. mexicana* shrank in response to a 10 days starvation period, but this reduction of body length was more pronounced in the cave ecotype. Both ecotypes reduced their metabolism as a response to starvation, but this effect did not differ significantly between ecotypes. Contrary to prediction, cave-dwelling *P. mexicana* clearly did not show lower metabolism; especially small cave fish even exhibited higher oxygen consumption than equal-sized surface fish. Presence of H_2S in this particular cave ecosystem is known to have led to the evolution of improved physiological detoxification mechanisms in cave-dwelling *P. mexicana*. As sulphide-detoxification is energetically costly (i.e., ATP-consuming), the continuous presence of H_2S during the evolutionary history of the cave ecotype may also have selected for increased ATP-production and thus, increased metabolism. Our study suggests that starvation adaptations may not be obligatory for cave life.

Keywords: cavefish, ecological speciation, food limitation, hydrogen sulphide, hypogean, oxygen uptake

Zusammenfassung: Höhlen werden oft als ressourcenarme Umgebung betrachtet, und bei den meisten bisher untersuchten Höhlenbewohnern wurden dementsprechend gesteigerte Hungerresistenzen und reduzierte Stoffwechselraten (Hypometabolismus) beschrieben. Wir haben beide Aspekte am neotropischen Lebendgebärenden Zahnkärpfling *Poecilia mexicana* aus einer ökologisch höchst produktiven Höhle untersucht, die giftiges (schwefelwasserstoffreiches) Wasser enthält, sowie an mehreren Populationen aus schwefelwasserstofffreien Oberflächenhabitaten. *P. mexicana* schrumpften als Reaktion auf eine zehntägige Hungerperiode, jedoch war die Reduktion der Körperlängen beim Höhlenökotyp stärker ausgeprägt. Beide Ökotypen reduzierten zudem ihren Stoffwechsel als Reaktion auf die Hungerperiode, unterschieden sich jedoch nicht signifikant in diesem Effekt. Entgegen der Erwartung zeigten höhlenbewohnende *P. mexicana* keine niedrigeren Stoffwechselraten. Insbesondere kleine Höhlenfische zeigten im Vergleich zu gleichgroßen Oberflächenfischen sogar einen höheren Sauerstoffverbrauch. Die Anwesenheit von Schwefelwasserstoff in diesem speziellen Höhlenökosystem hat bei höhlenbewohnenden *P. mexicana* zur Evolution von verbesserten physiologischen Detoxifikationsmechanismen

geführt. Da Schwefelentgiftung energetisch kostspielig ist (d.h., viel ATP verbraucht), könnte das kontinuierliche Vorhandensein von Schwefelwasserstoff während der Evolution des Höhlenökotyps für eine gesteigerte ATP-Produktion und somit einen erhöhten Stoffwechsel selektiert haben. Unsere Studie legt die Vermutung nahe, dass Hungeradaptationen für ein dauerhaftes Leben in Höhlen nicht bei allen Tieren zwingend erforderlich sind.

Schlüsselwörter: Höhlenfisch, ökologische Artbildung, Nahrungslimitierung, Schwefelwasserstoff, hypogäisch, Sauerstoffaufnahme

1. Introduction

Caves are traditionally described as being resource-limited habitats, because they lack photoautotrophic primary production and therefore have to rely on energy influx from adjacent surface habitats (Hüppop 2000, 2005, Poulson & Lavoie 2000). Allochthonous input is often sporadic and unpredictable, so that most obligate cave organisms (troglobites) have to withstand prolonged periods of resource shortage. Consequently, many troglobites show increased starvation resistance, e.g. due to reduced activity, lowered metabolism and/or increased amounts of storage fat tissues that help sustain periods of food shortage (crustaceans: Hervant et al. 1997, Hervant & Renault 2002, Simcic et al. 2005; fishes: Poulson 1963, Hüppop 1986, Pati & Agrawal 2002; amphibians: Hervant et al. 2000, 2001, Issartel et al. 2010; reviewed by Hüppop 2000, Hüppop 2005; but see Culver & Poulson 1971). Indeed, it has been hypothesized that lowered metabolic rates in general are an obligatory part of adaptation to cave life (Poulson & Lavoie 1969, Hüppop 2000, 2005, Issartel et al. 2010).

Compared to photosynthetically-based surface (epigean) ecosystems all cave ecosystems are probably energy-limited (Poulson & Lavoie 2000). However, some caves are far more energy-rich than others and potentially also provide ample resources for the animals inhabiting them. In subtropical and tropical caves, for example, allochthonous supply is greater than in temperate caves as a result of the greater biomass and uninterrupted production in epigean ecosystems (Mitchell 1969, Deharveng 2005), and this is especially true for sinkhole systems, such as the Cenotes of the Yucatan peninsula in Mexico (Schmitter-Soto et al. 2002). Furthermore, certain cave food webs are based on energy-rich guano deposits, where guano can stem from bats, birds and/or crickets (Gnaspini 2005). As guano is rich in undigested nutrients and energy (Gnaspini 2005, Fenolio et al. 2006), animal communities in guano-rich caves are usually highly complex and range from arthropods and other invertebrates to vertebrates (e.g. Poulson 1963, Ferreira & Martins 1999, Gnaspini 2005, Fenolio et al. 2006). Finally, caves can also be energy-rich resulting from chemolithoautotrophic bacteria, and probably the best understood chemolithoautotrophs are the sulphur oxidizers (Langecker et al. 1996, Sarbu et al. 1996, Palmer & Hill 2005, Engel et al. 2004; reviewed by Engel 2005, 2007). Reactive sulphur compounds in these systems are usually provided in the form of hydrogen sulphide (H_2S) and despite its toxicity sulphidic caves harbour an amazingly diverse fauna (Engel 2007).

Accordingly, Mitchell (1969) and Culver & Poulson (1971) already hypothesized that selection for lowered metabolic rates would be relaxed in energy-rich caves; a hypothesis that was repeated by Spicer (1998). Still, most physiological comparisons between closely related epigean and hypogean taxa have been performed in temperate regions on organisms from potentially food-limited caves (e.g., Poulson 1963, Hervant et al. 1997, 2000, 2001; Hervant & Renault 2002; Simcic et al. 2005, Issartel et al. 2010; but see Hüppop 1986, Pati & Agrawal 2002). At the same time, the hypothesis that hypogean organisms from energy-rich caves may not evolve reduced metabolism remains largely untested. One notable exception is a recent study by Salin et al. (2010) on hypogean and epigean *Astyanax fasciatus* (Cuvier, 1819) [also referred to as *A. mexicanus* (de Filippi, 1853)], in which the authors demonstrated that increased starvation resistance is not necessarily an ob-

ligate adaptation to cave life. Unfortunately, that study was conducted on fish of unknown origin, which were obtained from a professional breeder, so the results are not easily linked to any particular cave ecosystem of the more than 30 different Mexican caves harbouring troglobitic *Astyanax* that evolved in convergence (Mitchell et al. 1977, Wilkens 1988). Here, we present the first data on metabolism and starvation resistance between cave- versus surface-dwelling fish populations of well-known ecological and genetic background using Atlantic mollies, *Poecilia mexicana* Steindachner, 1863 (Teleostei: Poeciliidae), as model organisms.

Atlantic mollies are widespread along the Atlantic slope of Mexico (Miller 2005), and in tropical southern Mexico one population (the cave molly) has evolved in a sulphidic limestone cave, the Cueva del Azufre (a.k.a. Cueva de Villa Luz or Cueva de Las Sardinas; Gordon & Rosen 1962, Plath & Tobler 2010, Tobler & Plath 2011). As hydrogen sulphide is acutely toxic to metazoans (Bagarinao 1992, Grieshaber & Völkel 1998) and leads to extreme hypoxia in the water (Tobler et al. 2006, 2009b), cave mollies perform aquatic surface respiration (ASR) to exploit the more oxygenated (and thus less sulphidic) topmost layer of the water column (Plath et al. 2007b, Tobler et al. 2009a).

The cave molly system is intriguing for several reasons: (1) The Cueva del Azufre is one of only two sulphur caves known to be permanently inhabited by cavefish (Engel 2007). (2) Surface-dwelling ancestors of many cave organisms have become extinct, making a direct comparison of cave- and surface-dwelling forms of the same species impossible. In the cave molly complex (like in the aforementioned *A. fasciatus* complex), however, both epigean and hypogean forms exist within the same extant species. (3) Not only is this cave comparatively energy-rich because of its location in the tropics (Mitchell 1969, Deharveng 2005), but the presence of H_2S also allows for bacterial chemolithoautotrophic primary production (Langecker et al. 1996, Hose et al. 2000, Engel 2005, 2007). Furthermore, several species of bats roost in different cave chambers and deposit considerable amounts of bat guano (Gordon & Rosen 1962). (4) Finally, cave mollies have been well established as a model organism for incipient ecological speciation, and behavioural (Parzefall 2001, Plath et al. 2003, 2004, 2005, 2006), genetic (Plath et al. 2007a, 2010), morphological (Tobler et al. 2008, 2009b) and life-history adaptations (Riesch et al. 2009, 2010b, 2010c, 2011) to life in the Cueva del Azufre have been well documented. However, physiological adaptations in this system have thus far received little attention (for increased sulphide-tolerances in cave mollies see Peters et al. 1973; reanalysed in Plath & Tobler 2010).

In the present study, starvation resistance and metabolic rates in cave- and surface-dwelling populations of field-caught and lab-reared *P. mexicana* were compared with the following objectives: (1) to determine the metabolic adaptations (in terms of O_2-consumption) of *P. mexicana* to life in an extreme (i.e. permanently dark and toxic) habitat, (2) to test the hypothesis that organisms from energy-rich caves are under relaxed selection for lowered metabolic rates and increased starvation capabilities (Mitchell 1969, Culver & Poulson 1971, Spicer 1998), and (3) to evaluate if acute energy shortage (starvation) can be compensated by lowered metabolic rates.

2. Methods

2.1. Response to starvation in wild-caught fish

This experiment was conducted with field-caught *P. mexicana* that were collected in a sulphide-free river in the vicinity of the Cueva del Azufre (the Río Amatan) and in chamber V of the Cueva del Azufre (Gordon & Rosen 1962; for a map refer to Tobler et al. 2008) around January 10th, 2009. The Cueva del Azufre cave is characterized by several volcanic H_2S-rich springs feeding a creek that runs through the cave. Measurements of H_2S in the water read up to 300 µM (Tobler et al. 2006, 2008), which is acutely toxic for most animals (Smith et al. 1976, Smith & Gosselin 1979, Bagarinao 1992). At the same time, cave mollies live under hypoxia as oxygen concentrations are inversely correlated

with H_2S, ranging from 3.8% to 32% saturation (Tobler et al. 2006, Plath et al. 2010).

In the Cueva del Azufre, where the water is very shallow and low ceilings preclude seining, fish were caught with dip nets (13 × 14 cm, 1 mm meshwidth), while fish were caught using a seine (4 m long, 4 mm meshwidth) in the Río Amatan. Fish were transferred from the nets directly into aerated 40 l coolers and transported back to the University of Oklahoma. Upon arrival in the laboratory on January 14th, fish were transferred into 160 l, sulphide-free aquaria, and for 14 days were fed *ad libitum*-amounts of commercially available flake food. This two-week period allowed the fish to recover from the stress resulting from capture and transport.

Afterwards, a total of 80 fish (20 randomly selected males and females from each population) were introduced into 80 individual 8 l aquaria. Half of the fish (N=40; 10 males and 10 females from each population) were further assigned to a natural light/dark cycle and the other half (N=40) to permanent darkness. All 80 fish were then subjected to a 10 day starvation period. Since this experiment only lasted for ten days, water was not changed during the starvation period.

Originally, the starvation experiment was planned to run longer than 10 days. However, due to the unexpectedly high mortality of fish from both populations (see results) approximately one week into the experiment, the *ad hoc* decision was made to terminate the experiment after 10 days. To avoid similar mortality, the study on metabolic rates of starved fish (see below) was therefore run for only 7 days. Since no mortality was discovered this appears to have been the right decision.

Prior to the beginning of the experiment and immediately afterwards, standard length (L_s) [mm] and wet body mass (W) [g] were recorded for each individual, and the condition factor according to Williams (2000) was calculated: $K = 100{,}000\ W^{*}L_s^{-3}$.

In three separate full factorial repeated measures ANOVAs, (*a*) log-transformed standard length [mm], (*b*) log-transformed wet weight [g], and (*c*) log-transformed condition factor [g/mm^3] were compared before and after the starvation period (rm). In all models 'population' (2 levels: cave molly vs. surface molly), 'sex' (2 levels: male vs. female), and 'light condition' (2 levels: complete darkness vs. light/dark cycle) were coded as factors.

2.2. O_2-consumption in common-garden reared fish

Respirometric measurements were conducted in the laboratory of the University of Frankfurt, Department of Animal Physiology, between January and February 2010. Experimental animals were descendants of individuals stemming from three divergent populations that were collected between 1995 and 2010: The first surface population originated from the Río Oxolotán (N=22), another tropical river of the Río Grijalva drainage in close proximity to the Cueva del Azufre and the Río Amatan (Tobler et al. 2006, 2008). Animals for the second surface populations were originally sampled in the Río Panuco drainage near the city of Tampico in eastern Mexico (N=25). This population is approximately 800 km to the north of the cave and surface populations from southern Mexico (see map in Riesch et al. 2010b) and was chosen as an independent reference to evaluate within-species variation of epigean *P. mexicana*. The third population was the cave form of *P. mexicana* from chamber XIII (Gordon & Rosen 1962; N=33). All test fish (including the cave form) were reared under the same conditions, i.e. under a 12:12 hours light:dark regime, in sulphide-free water (water quality was maintained by partial changes every 1-2 weeks), and were fed *ad libitum*-amounts of flake food, fish food tablets and occasionally live *Artemia* spp. nauplii, *Chironomus* larvae or water flees.

O_2-consumption of fish was measured in an open-flow respirometry system, consisting of a cylindrical respirometer chamber (diameter 5.7 cm, length 22 cm, volume 0.56 l), placed in a plastic fish tank (56 x 25 x 15.5 cm) and attached to a tubing system with O_2-saturated water of 25 °C running at a constant flow rate of 2.5 l/h (Köhler et al. 2011). Flow rate was con-

tinuously monitored and controlled by a valve and a flow-meter [ROTA Type K12/G1831/77 (WEHR–2), accuracy ±1.6%]. Directly before and after the respirometry chamber, O_2-content was measured by two oxygen electrodes (Oxi 315i, WTW; accuracy: ±0.5%), which were built into the system in sealed custom-made cuvettes. Oxygen electrodes were calibrated before each measurement against water vapour saturated air by inserting the sensor in the OxiCal-SL container provided by the manufacturer. Magnetic stirrers kept flow conditions in the measuring cuvettes constant. The whole setup was covered with brown cardboard, such that any disturbance from the outside was reduced to a minimum, however, light was provided by overhead fixtures (room illumination).

Following established protocols for this species (Köhler et al. 2011), animals were placed in the respirometry chamber and allowed to settle for 20 min in order to obtain resting metabolic rates. Oxygen consumption rates were then recorded over a duration of 10 minutes, provided steady-state readings from the electrodes. Afterwards, fish were weighed to the nearest 0.1 g using a Sartorius PT 600 scale (accuracy: ±0.1%). Measurements were run daily between 10:00 and 15:00 h (well after the experimental animals had been fed) to ensure comparable conditions of feeding status and diurnal activity rhythm of the test subjects.

Log-transformed O_2-consumption rates [ml h^{-1}] were compared in a univariate ANCOVA, while using 'population' and 'sex' as factors and log-transformed body mass [g] as a covariate to account for the well-known positive correlation between body mass and O_2-consumption (Clarke & Johnston 1999, White et al. 2006). Non-significant interaction effects were eliminated starting with highest-level interactions.

2.3. O_2-consumption before and after starvation in common-garden reared fish

Reduced metabolic rates are a typical response to starvation in various animals (Hickman 1959, Beamish 1964, Wieser et al. 1992), and cave mollies theoretically might show a stronger decrease in metabolism in response to starvation than surface-dwelling fish, so lower metabolism in the cave form might only be detectible after starvation. To test this hypothesis, O_2-consumption before and after seven days of food deprivation were analysed in one population from the Cueva del Azufre (chamber XIII, N=9) and the two surface-dwelling populations of *P. mexicana* from the previous analysis: Río Oxolotán (N=7) and Tampico (N=8). Prior to testing fish were isolated in groups of 2-3 individuals in 10 l tanks under a 12:12 hours illumination cycle. The tanks were well aerated and kept at a constant temperature of 25 °C. To sustain water quality, 50% of water was changed twice during the 7 day period. After the first O_2-measurement test fishes obtained no food for seven days and were then tested again for their O_2-consumption. Tests took place at the same time of day as the experiments with well-fed individuals.

Sample sizes were comparatively small in this experiment, precluding an analysis as outlined in the previous section (i.e., ANCOVA with various interaction effects in the model). Therefore, log-transformed O_2-consumption rates [ml h^{-1}] before and after the starvation period (rm) were compared in a full factorial repeated-measures general linear model (rmGLM), while 'population' was included as a factor and log-transformed body mass [g] as a covariate (Clarke & Johnston 1999, White et al. 2006).

3. Results

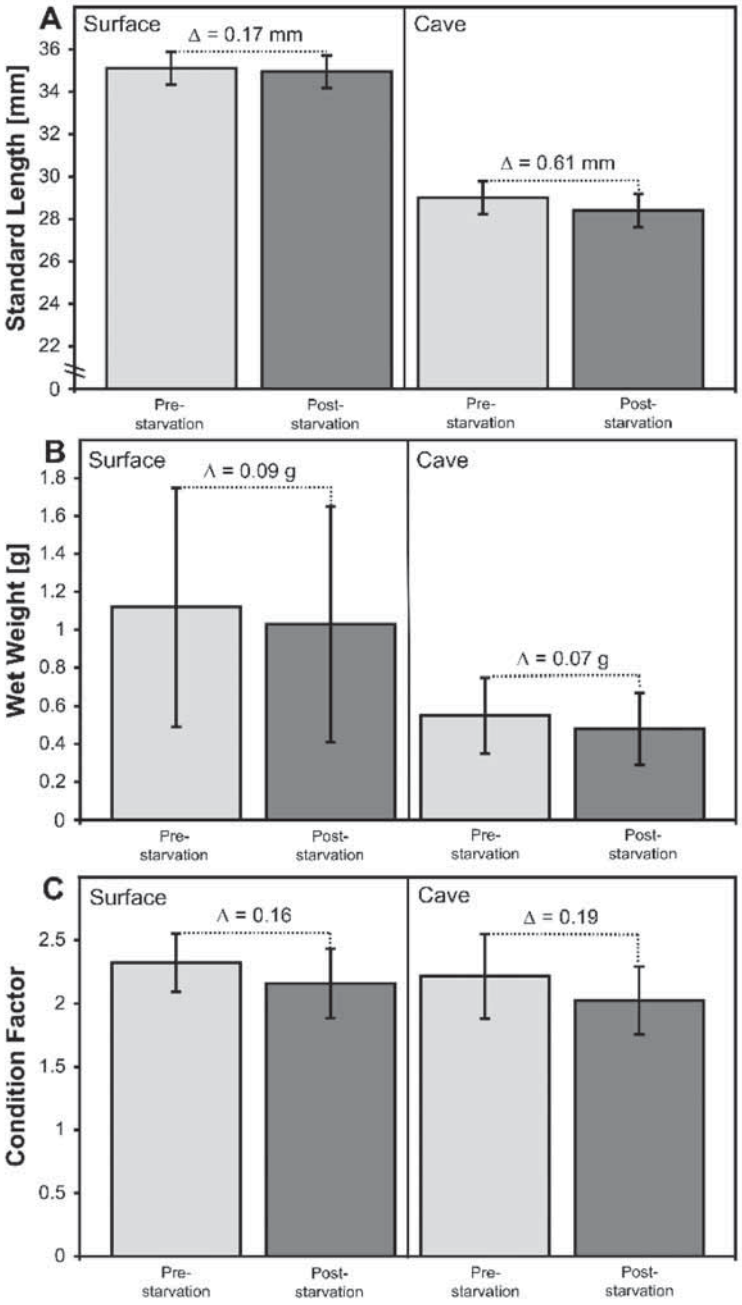
3.1. Response to starvation in wild-caught fish

A total of 15 animals (18.8%) died during the starvation experiment [cave mollies: N=8 (20%); surface mollies: N=7 (17.5%)], but the two populations did not differ in food-stress related mortality (binary logistic regression: -2 log likelihood=77.13, *Wald*=0.082, *df*=1, P=0.85). Starvation (i.e., the repeated measurement) had a significant effect on standard length, indicating that fish of both ecotypes decreased in length over the 10 day starvation period (tab. 1A, fig. 1A). Standard length in cave fish from the

Tab. 1: Results from full-factorial repeated-measures ANOVA on (*a*) log_{10}-transformed standard length, both prior to and after a ten day starvation experiment (rm), (*b*) log_{10}-transformed wet weight and (*c*) log_{10}-transformed condition factor. The model included 'population', 'sex', and 'treatment' as between subject factors. Significant effects are in bold.

Tab. 1: Ergebnisse vollfaktorieller Varianzanalysen mit Messwiederholungen zu (*a*) log_{10}-transformierter Standardlänge (mm), gemessen sowohl vor als auch nach der zehntägigen Hungerperiode (rm), (*b*) log_{10}-transformiertem Nassgewicht und (*c*) log_{10}-transformiertem Konditionsfaktor. Das Modell beinhaltete 'Population', 'Geschlecht' (Sex) und 'Versuchsbedingung' (Treatment) als Faktoren. Signifikante Effekte sind fett hervorgehoben.

Effects		*df*	Mean Square	*F*	*P*
(*a*) Standard length					
Within-subjects	**Rm**	**1**	**0.001**	**13.837**	**<0.001**
	Rm * pop	**1**	**<0.001**	**5.027**	**0.029**
	Rm * light	**1**	**0.001**	**13.061**	**0.001**
	Rm * sex	1	<0.001	0.879	0.352
	Rm * pop * light	1	<0.001	0.023	0.880
	Rm * pop * sex	1	<0.001	1.547	0.219
	Rm * light * sex	1	<0.001	1.084	0.302
	Rm * pop * light * sex	1	<0.001	0.149	0.701
	Error (rm)	57	<0.001		
Between-subjects	**Pop**	**1**	**0.223**	**36.894**	**<0.001**
	Light	1	0.009	1.562	0.216
	Sex	**1**	**0.058**	**9.622**	**0.003**
	Pop * light	1	0.014	2.387	0.128
	Pop * sex	1	0.008	1.393	0.243
	Light * sex	1	0.010	1.652	0.204
	Pop * light * sex	1	<0.001	0.074	0.304
	Error	57	0.006		
(*b*) Wet weight					
Within-subjects	**Rm**	**1**	**0.084**	**220.785**	**<0.001**
	Rm * pop	**1**	**<0.001**	**10.629**	**0.002**
	Rm * light	1	<0.001	0.898	0.347
	Rm * sex	1	<0.001	<0.001	0.993
	Rm * pop * light	**1**	**0.002**	**5.042**	**0.029**
	Rm * pop * sex	1	<0.001	0.311	0.580
	Rm * light * sex	1	0.001	1.837	0.181
	Rm * pop * light * sex	1	<0.001	0.329	0.568
	Error (rm)	57	<0.001		
Between-subjects	**Pop**	**1**	**2.436**	**38.292**	**<0.001**
	Light	1	0.068	1.069	0.305
	Sex	**1**	**1.072**	**16.845**	**<0.001**
	Pop * light	1	0.204	3.199	0.079
	Pop * sex	1	0.059	0.933	0.338
	Light * sex	1	0.085	1.332	0.253
	Pop * light * sex	1	0.006	0.091	0.763
	Error	57	0.064		
(*c*) Condition factor					
Within-subjects	**Rm**	**1**	**0.038**	**42.015**	**<0.001**
	Rm * pop	1	<0.001	0.037	0.849
	Rm * light	**1**	**0.006**	**6.258**	**0.015**
	Rm * sex	1	0.001	0.646	0.425
	Rm * pop * light	1	0.002	2.544	0.116
	Rm * pop * sex	1	0.002	2.066	0.156
	Rm * light * sex	1	<0.001	<0.001	0.988
	Rm * pop * light * sex	1	<0.001	0.500	0.482
	Error (rm)	57	0.001		
Between-subjects	**Pop**	**1**	**0.021**	**5.701**	**0.020**
	Light	1	0.001	0.259	0.613
	Sex	**1**	**0.097**	**26.683**	**<0.001**
	Pop * light	1	0.008	2.262	0.138
	Pop * sex	1	0.001	0.275	0.602
	Light * sex	1	<0.001	0.020	0.887
	Pop * light * sex	1	<0.001	0.046	0.831
	Error	57	0.004		



Figs. 1A-C: Results from a 10 day starvation experiment using wild-caught *P. mexicana* from Río Amatan (surface, left) and Cueva del Azufre, chamber V (cave, right). Mean (±SD) of **A** standard length, **B** wet weight, and **C** condition factor. Sexes were pooled because they did not differ in their response to starvation (tab. 1).
Abb. 1A-C: Ergebnisse eines zehntägigen Hungerexperimentes an wildgefangenen *P. mexicana* aus dem Río Amatan (surface, links) und der Cueva del Azufre, Kammer V (cave, rechts). Dargestellt sind Mittelwert (±Standardabweichung) **A** der Standardlänge, **B** des Nassgewichts und **C** des Konditionsfaktors. Geschlechter wurden zusammen analysiert, da sie sich nicht in ihrer Reaktion auf die Hungerperiode unterschieden (Tab. 1).

Cueva del Azufre dropped from 28.81±3.23 mm (mean±SD) to 28.25±3.03 mm (1.94% length decrease; paired *t*-test: t_{31}=3.356, *P*=0.002), while surface mollies from Río Amatan decreased in length from 35.24±5.90 mm to 35.00±5.88 mm (0.57% length decrease; t_{32}=1.543, *P*=0.133). This difference between ecotypes was supported by the significant interaction term 'starvation by population' on L_s (tab. 1A), indicating that cave mollies exhibited a stronger decrease in size than surface mollies (fig. 1A). Also, 'starvation by light condition' was statistically significant, because both ecotypes 'shrunk' more in light (2.34% L_s-decrease across ecotypes, from 33.39±6.22 mm to 32.61±6.20 mm, t_{32}=5.520, *P*<0.001), but showed almost no full body shrinkage in darkness (0.08%, from 30.72±4.93 mm to 30.72±5.22 mm, t_{31}<0.001, *P*=1.000).

Starvation also had a significant influence on wet weight, indicating that both ecotypes lost body mass during the 10 day starvation period (tab. 1B, fig. 1B). In cave mollies, wet weight dropped from 0.55±0.20 g to 0.48±0.19 g (12.73% weight decrease; paired *t*-test: t_{31}=12.555, *P*<0.001), while it dropped from 1.12±0.63 g to 1.03±0.62 g in surface mollies (8.04% weight decrease; paired *t*-test: t_{32}=10.472, *P*<0.001). This difference in weight loss between ecotypes was supported by the interaction term 'starvation by population' (tab. 1B), indicating that cave mollies lost more weight than surface mollies (fig. 1B). However, the three-way interaction of 'starvation by population by light condition' was also significant (tab. 1B), because cave mollies lost more weight in darkness (weight decrease, light: 12.24%; darkness: 13.91%), while surface mollies lost more weight in light (light: 8.94%; darkness: 5.72%).

Starvation further significantly lowered condition factors in both ecotypes over the 10 day starvation period (tab. 1B, fig. 1B). However, there was no indication that one of the two ecotypes reduced body condition more than the other (see non-significant interaction effect of 'starvation by ecotype' in tab. 1B). In fact, condition factor decreased in cave mollies from 2.22±0.33 g/mm^3 to 2.03±0.27 g/mm^3 (i.e. by 8.56%; t_{31}=3.865, *P*=0.001) and in surface mollies from 2.32±0.23 g/mm^3 to 2.16±0.27 g/mm^3 (6.90%; t_{32}=5.216, *P*<0.001; fig. 1B). Finally, 'starvation by light condition' had a significant impact on body condition, as condition dropped to a significantly larger extent in darkness (light: 4.93% condition factor-decrease across ecotypes, from 2.23±0.27 g/mm^3 to 2.12±0.28 g/mm^3, t_{32}=3.293, *P*=0.003; darkness: 10.82%, from 2.31±0.30 g/mm^3 to 2.06±0.27 g/mm^3, t_{31}=5.573, *P*<0.001; tab. 1B).

3.2. O_2-consumption in common-garden reared fish

In the ANCOVA analysing oxygen consumption, neither a statistically significant effect of the factor 'sex' nor any significant interaction effects involving 'sex' were detected (tab. 2). Data depicted in figure 2 and linear regressions used in the analyses described below are, therefore, based on combined data-sets for males and females.
The covariate (body mass) had a significant effect on O_2-consumption (tab. 2), and metabolic rates generally increased with increasing body size (fig. 2). Even though test fish were selected from the stock tanks in such a way that a maximum range in body size and mass was available, body mass in this study was distinctly lower in cave fish (size range: 0.21-5.60 g; mean±SD: 1.32±1.32 g) than in the two surface populations (Tampico: 0.50-7.20 g; mean±SD: 2.91±1.99 g; Río Oxolotán: 0.40-13.50 g; mean±SD: 3.39±3.62 g), which matches size differences in natural populations.

ANCOVA also detected a statistically significant difference among populations (tab. 2), as mass-specific metabolic rates were highest in the cave population (fig. 2A). However, there was also a significant interaction effect of 'population by body size' in the ANCOVA (tab. 2), suggesting that the observed population difference was largely driven by different slopes for the linear regressions between log-transformed body mass and log-transformed oxygen consumption (fig. 2B). Indeed, while both surface populations showed a clear-cut pattern of oxy-

Tab. 2: Results from a univariate ANOVA (R^2=0.73) using log-transformed O_2-consumption as dependent variable, 'population' and 'sex' as fixed factors and log-transformed body mass as a covariate. The three-way interaction term was not significant (Mean Square=0.002, $F_{2,68}$=0.100, P=0.910) and thus removed from the analysis. Significant P-values are in bold.

Tab. 2: Ergebnisse einer univariaten Varianzanalyse (R^2=0.73) mit log-transformiertem Sauerstoffverbrauch als abhängiger Variable, 'Population' und 'Geschlecht' (Sex) als Faktoren und log-transformiertem Körpergewicht als Kovariate. Dreifachinteraktionen waren nicht signifikant (Mean Square=0.002, $F_{2,68}$=0.100, P=0.910) und wurden daher aus der Analyse ausgeschlossen. Signifikante P-Werte sind fett hervorgehoben.

Effect	*df*	Mean Square	*F*	*P*
Population	**2**	**0.233**	**9.627**	**<0.001**
Sex	1	0.001	0.045	0.833
log (body mass)	**1**	**1.914**	**79.131**	**<0.001**
Population * sex	2	0.020	0.839	0.437
Population * log (body mass)	**2**	**0.166**	**6.881**	**0.002**
Sex * log (body mass)	1	0.033	1.351	0.249
Error	70	0.024		

gen consumption increasing in a linear fashion with increasing body mass, several small-bodied cave fish showed unexpectedly high metabolic rates along with largely increased variance in this size-class (fig. 2B). This led to a distinctly lower value for the slope of the linear regression (i.e. allometric exponents) between log-transformed body mass and log-transformed oxygen consumption in cave fish (Tampico: slope=0.641, R^2=0.75; Río Oxolotán: slope=0.678, R^2=0.84; cave form: slope=0.549, R^2=0.19). In summary, the statistical analysis could clearly rule out that cave fish have lowered metabolic rates (figs. 2A, B), while the apparent increase in metabolic rates in this population appears to be driven primarily by small fish showing unexpectedly high metabolic rates.

3.3. O_2-consumption before and after starvation in common-garden reared fish

Fish of all three populations were lighter after the 7-day starvation period (paired *t*-tests, Tampico: t_7=5.167, P=0.001; Río Oxolotán: t_6=3.860, P=0.008, cave population: t_8=7.560, P<0.001). Body mass in fish from Tampico dropped from 4.49±1.70 g (mean±SD) to 4.11±1.56 g (i.e. 91.7% of the original body mass). In fish from the Río Oxolotán, body mass dropped from 5.16±2.96 g to 4.49±2.59 g

Tab. 3: Results from a full factorial repeated-measures GLM on 'log-transformed O_2-consumption' prior to and after a seven day starvation period (rm). 'Population' was the between-subjects factor, and 'body mass' (log-transformed) was included as a covariate. Significant P-values are in bold.

Tab. 3: Ergebnisse eines vollfaktoriellen Generellen Linearen Modells (GLM) mit MesswiEderholungen zur Analyse von 'log-transformiertem Sauerstoffverbrauch' vor und nach einer siebentägigen Hungerperiode (rm). 'Population' war als Faktor und 'log-transformiertes Körpergewicht' als Kovariate kodiert. Signifikante P-Werte sind fett hervorgehoben.

Effects		*df*	Mean Square	*F*	*P*
Within-subjects	**Rm**	**1**	**0.077**	**5.142**	**0.035**
	Rm * log (body mass)	1	0.007	0.458	0.506
	Rm * pop	2	0.002	0.143	0.867
	Error (rm)	20	0.015		
Between-subjects	**Log (body mass)**	**1**	**0.642**	**35.063**	**<0.001**
	Pop	2	0.023	1.266	0.304
	Error	20	0.018		

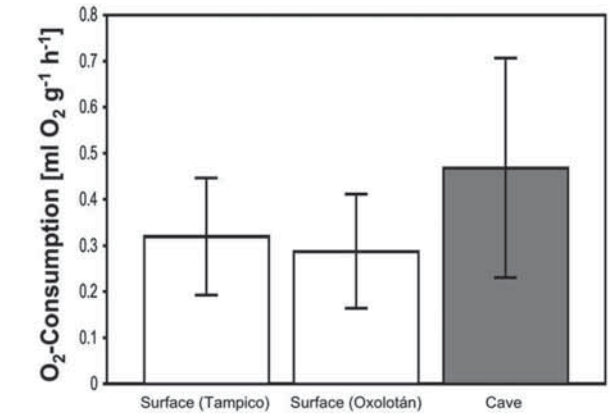
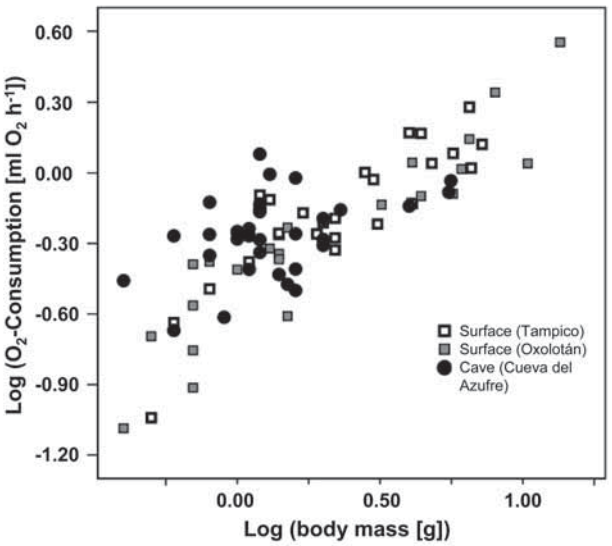


Fig. 2A



Figs. 2 A and B: A Mean (±SD) oxygen consumption [ml O_2 g^{-1} h^{-1}], and **B** relationship between log_{10}-transformed body mass [g] and log_{10}-transformed oxygen consumption [ml O_2 h^{-1}] of laboratory-reared *P. mexicana* from three different populations.

Abb. 2 A und B: A Mittelwert (±Standardabweichung) des Sauerstoffverbrauchs [ml O_2 g^{-1} h^{-1}] und **B** das Verhältnis zwischen log_{10}-transformiertem Körpergewicht [g] und log_{10}-transformiertem Sauerstoffverbrauch [ml O_2 h^{-1}] von laboraufgezogenen *P. mexicana* aus drei verschiedenen Populationen.

(85.7%), and in cave fish from the Cueva del Azufre, body mass dropped from 2.57±0.55 g to 2.31±0.49 g (90.1%). Hence, mean values from before and after the starvation treatment were included as a covariate in the rmANCOVA. Just like in the previous analysis, log-transformed body mass had a significant effect on oxygen consumption (tab. 3), and metabolic rates generally increased with increasing body mass.

Metabolic rates decreased during the 7 day starvation period in all populations examined (see 'repeated measurement' in tab. 3). However, there was no statistically significant difference among populations in their response to starvation (see 'repeated measurement by population' in tab. 3; fig. 3 for mass-specific metabolic rates). After 7 days of starvation, mass-specific metabolic rates dropped to 75.2% in the Tampico

population, 89.4% in surface fish from the Río Oxolotán, and 79.6% in cave fish from the Cueva del Azufre.

4. Discussion

Contrary to most studies that compared closely related epigean and hypogean taxa (reviews in Hüppop 2000, 2005) no evidence for increased starvation resistance or lowered metabolic rates in the cave molly was detected. In fact, cave mollies showed a stronger reduction in body length, weight and condition factor as a result of starvation than epigean *Poecilia mexicana*. Furthermore, cave mollies had the highest metabolic rates of all three *P. mexicana* populations compared, even though this effect appeared to be largely driven by particularly high metabolism of smaller cave molly individuals compared to equal-sized surface fish.

4.1. Phenotypic flexibility and full-body shrinkage

Phenotypic flexibility is the ability of an individual organism to induce reversible variation as a response to environmental change (Piersma & Drent 2003). In physiology, this concept is probably best encompassed by allostasis (i.e. "stability through change"; Sterling 2004, Korte et al. 2005, Schreck 2010). Size-reduction of certain body parts or organs, for example, is a common response to low food availability in a variety of invertebrates and vertebrates, but the most drastic response to local food shortage happens when organisms become smaller. This has been described in a variety of invertebrates (i.e. sea urchins and crustaceans; Piersma & Drent 2003) and also in vertebrates, such as the Brazilian cave catfishes *Trichomycterus itacarambiensis* Trajano & de Pinna, 1996 (Trajano 1997) and *Ancistrus cryptophthalmus* Reis, 1987 (Trajano & Bichuette 2007), the Ozark cavefish *Amblyopsis rosae* (Eigenmann, 1898) (Brown & Johnson 2001) and the marine iguana *Amblyrhynchus cristatus* Bell, 1825 (Wikelski & Thom 2000). However, follow-up studies on other vertebrates have often been unsuccessful in replicating similar trends (Madsen & Shine 2001, Luiselli 2005).

Why would *P. mexicana* get shorter as a response to starvation? Wikelski & Thom (2000) demonstrated that in marine iguanas length-reduction during El Niño events led to increased survival and thus argued that 'shrinkage' represents an adaptive response to nutritional stress. Likewise, by decreasing body size (i.e. length and weight), starved *P. mexicana* reduce their absolute energy expenditure (Wikelski & Thom 2000), in other words, body size reduction could be an allostatic response to re-establish homeostasis (Schreck 2010). This would also explain why cave mollies 'shrunk' to a larger extent than surface mollies, because they would have to compensate for having a higher metabolism by reducing body length to a larger extent. Thus, length reduction as a response to low food availability may be more widespread among vertebrates than previously suspected (Piersma & Drent 2003).

4.2. Cave organisms and metabolic economy

Over the years, several hypotheses have been proposed to explain the convergent patterns of evolution observed in many cave animals. The Food-Limitation Hypothesis (Poulson 1963), for example, assumes that the standard metabolic rate of a troglobite should reflect the evolutionary time a species has spent in caves. In other words, metabolic rates should be lower in evolutionarily old compared to younger troglobites. Even though it is so far unknown how long ago cave mollies colonized the Cueva del Azufre, the prediction from the food-limitation hypothesis would have been at least a slightly lower metabolic rate in cave mollies relative to epigean *P. mexicana*. However, the present study could clearly reject this prediction, even after a one week starvation period (fig. 2), which is strong evidence against this hypothesis in its original form. On the other hand, the present results are congruent with a variant of the Food-Limitation Hypothesis proposed by Poulson & Culver (1971): if food/energy is not

limited, then there should also be no selection for metabolic economy.

4.3. Metabolic economy in the cave molly

Our study demonstrates a higher metabolic rate and lower starvation resistance in a cave-dweller compared to its surface-dwelling relatives (see also Culver & Poulson 1971). A closer inspection of fig. 2, however, reveals a slightly surprising pattern: while small cave mollies clearly have a higher metabolism, the pattern seems to disappear in larger specimens. This pattern is tentatively interpreted as an artefact of conducting this particular experiment with lab-reared fish. If one compared the mass of all field-caught fish used in the present study (log_{10}-transformed body mass, surface mollies: -0.01±0.24 g (mean±SD), min=-0.48 g, max=0.55 g; cave mollies: -0.29±0.15 g, min=-0.60 g, max=0.06 g) with those from this particular experiment, it is becomes evident that the left half of fig. 2 (i.e., cave mollies of low body mass, having a higher metabolic rate) is representative for the field-caught specimens. In fact, the left half (i.e. low mass-range) of figure 2 also best represents the mass–range of field-caught fish from these two environments that we sampled for previous studies (Riesch et al. 2010b, 2011, Riesch unpubl. data). This demonstrates that cave and surface mollies in natural populations rarely live long enough to achieve a size similar to those that contributed to the pattern on the right side (i.e. upper mass range) of fig. 2. Hence, caution is required when interpreting the pattern revealed by larger fish in the present analysis, as this could, for example, be confounded by a secondarily reduced metabolism due to senescence (e.g. Medawar 1952, Williams 1957).

How can elevated metabolism in cave mollies be explained? It is hypothesised that this represents an adaptation to hydrogen sulphide, as coping with toxicants requires costly (i.e. time- and energy-consuming) behavioural and physiological adaptations (e.g. Calow 1989, Sibly & Calow 1989, Schreck 2010). At present, the exact physiological mechanisms that enable *P. mexicana* to survive the toxic conditions are not yet known. However, several species capable of sulphide oxidation exhibit increased oxygen consumption because large amounts of oxygen are required for hydrogen sulphide detoxification pathways (Bagarinao 1992, Grieshaber & Völkel 1998). Future research will have to investigate this further in *P. mexicana* and other extremophile poeciliids, but this interpretation is also congruent with the lower body condition (measured as percent body fat) and lower lean weights found in wild-caught and lab-reared cave mollies relative to surface mollies of the same size (Tobler 2008, Riesch et al. 2010b, 2011).

4.4. Ecological and evolutionary implications

Not only did wild-caught cave mollies perform worse than surface mollies when exposed to a 10 day starvation period, but cave mollies that had been raised for several generations in the laboratory also showed consistently higher oxygen consumption rates than surface mollies under our non-sulphidic experimental conditions. This could indicate that the physiological adaptations to sulphide toxicity are genetically inherited and that cave mollies might not be able to simply 'switch' detoxification pathways off when in non-toxic waters. Given that cave mollies have now survived for several thousand generations in the constant presence of hydrogen sulphide and that maintaining phenotypic plasticity can be energetically costly (DeWitt et al. 1998, Relyea 2002), such a scenario is congruent with the hypothesis of 'genetic assimilation' (Pigliucci et al. 2006). However, it is important to note that the present study was performed in an H_2S-free environment. To unequivocally test our hypothesis, future studies will have to attempt to compare metabolic rates of cave and surface mollies in the presence and absence of H_2S – a challenging task, because H_2S is also highly toxic to humans (Bagarinao 1992, Grieshaber & Völkel 1998) and non-adapted *P. mexicana* have been demonstrated to die within hours of exposure to hydrogen sulphide levels present throughout the cave (Tobler et al. 2009b).

4.5. Conclusions

The present study exemplifies that starvation resistance and lowered metabolic rates are not always obligatory, essential adaptations for cave life (Poulson & White 1969, Hüppop 2000, 2005). Rather, they appear to be adaptations to the low food environments experienced in most temperate caves (Mitchell 1969, Culver & Poulson 1971, Spicer 1998). It seems reasonable to argue that increased metabolic rates in cave mollies could be explained by the presence of high levels of toxic hydrogen sulphide in the Cueva del Azufre (Hose et al. 2000, Tobler et al. 2006), that make this cave unique compared to most other cave ecosystems. Future investigations are warranted looking into a mechanistic explanation for this interesting pattern, and further studies are underway that investigate cellular ultra-structure and H_2S-detoxification (e.g. via transcriptomics).

In this regard, the present study further indicates the importance of hydrogen sulphide as a strong selective force that requires (costly) adaptations for long-term survival in sulphidic habitats and thus as a driver for ecological divergence (Bagarinao 1992, Grieshaber & Völkel 1998, Plath et al. 2007a, Tobler et al. 2008, 2009a, 2009b, Riesch et al. 2009, 2010a, 2010b, 2010c, 2011). Finally, the present study stresses the importance of evaluating any given study organism within the full breadth of its ecological environment when trying to interpret its physiological capacities.

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