

# Sperm production in an extremophile fish, the cave molly (*Poecilia mexicana*, Poeciliidae, Teleostei)

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**Abstract** A prominent trade-off in life history theory and evolution balances the costs of reproduction with those of basic somatic needs. Hence, reproductive efforts may be reduced in environments where additional energy is required for somatic maintenance. Here, we investigated male sperm stores in Atlantic mollies (*Poecilia mexicana*) from a sulfidic cave and several sulfidic and non-sulfidic surface habitats. We found significant differences among populations in the number of sperm stripped per male, which was also correlated with differences in gonad weights. The largest sperm stores were detected in males from non-sulfidic surface creeks,

while males from a partially sulfidic surface system had lower sperm counts, and males from completely sulfidic systems, surface as well as subterranean, had even fewer available sperm. We conclude that the extreme environmental conditions in sulfidic habitats appear to constrain male sperm production, since hydrogen sulfide as a naturally occurring toxin requires energy-demanding adaptations. Furthermore, we examined sperm counts of lab-reared cave and surface mollies in response to energy limitation. Males from stock populations were placed under high and low food treatments for a 2-week period and then stripped of sperm. Sperm counts of surface mollies tended to be reduced by low food availability, whereas sperm counts of cave mollies did not significantly vary between food treatments, which likely points towards a higher starvation resistance in cave mollies.

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## Introduction

Each individual must balance the energy allotment required for basic somatic needs and those required for reproductive efforts (Bell and Koufopanou 1986; Stearns 1989). Such trade-offs may differ vastly across environments, and individuals inhabiting extreme environmental conditions are expected to

invest more energy in somatic maintenance than those residing in less harsh habitats (Sibly and Calow 1989; Celentano and Defeo 2006). Extreme environmental conditions are defined as requiring, of any organism tolerating them, costly adaptations that are absent in most related species (Townsend et al. 2003). For example, hydrogen sulfide ( $H_2S$ ) is acutely toxic and therefore unambiguously an extreme environmental factor for all animal life (Evans 1967; Theede 1973; Smith et al. 1976; Bararinao and Vetter 1989; Grieshaber and Völkel 1998). Most metazoans known to inhabit aquatic sulfidic habitats are invertebrates (Grieshaber and Völkel 1998; McMullin et al. 2000), while only very few freshwater fishes are known to tolerate the toxic effects of  $H_2S$  for long periods and thus permanently inhabit sulfidic habitats. A unique freshwater system that is rich in  $H_2S$  was described in southern Mexico. This sulfidic system is located about 30 km south of Teapa, Tabasco, where several  $H_2S$ -containing springs feed a creek that drains a cave (Cueva del Azufre; Gordon and Rosen 1962; Tobler et al. 2006). Compared to the surrounding surface habitats, the cave harbors a reduced fish fauna in that it is solely inhabited by a distinct population of the widespread Atlantic molly, *P. mexicana* (the cave molly; Gordon and Rosen 1962; Tobler et al. 2006).

Extensive research has been carried out on the cave molly (Parzefall 2001; Plath et al. 2004, 2005, 2006a, 2007); however, most studies have concentrated on behavioral and morphological adaptations of cave mollies to darkness (reviewed in Parzefall 2001). Hence, little is known about the reproductive biology of the cave molly, especially with regard to how the extreme environmental conditions (i.e., the presence of  $H_2S$  and absence of light) act as limiting factors (Tobler et al. 2006).

Experimental evidence suggests the immediate survival of cave mollies is dependent on access to the air–water interface where they perform aquatic surface respiration (ASR; Plath et al. *in press*), a time- and energy-demanding behavior (Weber and Kramer 1983; Kramer 1987). Cave mollies provided with energy-rich food had a higher chance of survival especially when access to the water surface was denied, indicating that energy availability may be

limited (Plath et al. *in press*). In accordance with this idea, cave mollies show a dramatically reduced body condition compared to surface-dwelling mollies from non-sulfidic habitats (Plath et al. 2005; Tobler et al. 2006), suggesting either food resources (i.e., detritus, sulfur-bacteria, chironomid larvae, and small aquatic snails; Langecker et al. 1996; M. Tobler, unpubl. data) are of poor quality or low abundance, and/or surviving under the extreme conditions is energetically demanding.

We assessed the relationship between low body condition and an important aspect of males' life history, namely sperm production, across populations living in vastly different environments. Differences in sperm production among populations or species are usually thought to be driven by differences in sperm competition across different mating systems, and higher levels of sperm competition are usually correlated with higher investments in sperm production (Birkhead and Møller 1998). Although sperm production is considered far less energy-demanding than maternal investment (Bateman 1948; Trivers 1972), it is not cost-neutral, and indeed sperm-limitation has been found in some species, e.g., due to sperm-depletion after copulation (Nakatsuru and Kramer 1982; Birkhead 1991; Wedell et al. 2002; Smith and Reichard 2005). In addition, harsh environmental conditions may also affect sperm production, because males may need to reallocate more energy to ensure their immediate survival (e.g., ASR and physiological detoxification). However, observational evidence for limited sperm availability in harsh environments is scarce.

Here, we used a comparative approach to quantify sperm availability of *P. mexicana* between different populations. First we took advantage of the unique system consisting of several differentiated populations of *P. mexicana* inhabiting proximate, but vastly different habitats within a small area in and around the Cueva del Azufre. We compared male sperm stores in populations from non-sulfidic, normoxic surface habitats, sulfidic surface habitats, and the sulfidic, hypoxic cave (Tobler et al. 2006; Plath et al. 2007). Second, we compared the effects of energy availability in the absence of  $H_2S$  on sperm production in laboratory-reared surface and cave mollies under high and low food treatments.

## Materials and methods

### Field study

*Poecilia mexicana* were collected in cave chambers V and X of the sulfidic Cueva del Azufre near the village of Tapijulapa in Tabasco, Mexico [17°26.5'N, 92°46.5'W; see Gordon and Rosen (1962) for denomination of cave chambers] in January 2006. Measurements of H<sub>2</sub>S revealed high concentrations in cave chamber V (2–32 µM/l; Tobler et al. 2006). The most upstream sulfidic springs are located in cave chamber X and H<sub>2</sub>S concentrations are generally high (2–320 µM/l), however, fish are able to move into non-sulfidic water upstream of the first sulfidic springhead within a few meters (Tobler et al. 2006). Thus, we consider cave chamber X a habitat with overall moderate H<sub>2</sub>S concentration, despite the high measurements directly at the sulfidic springs. In addition, we sampled two sulfidic surface habitats: El Azufre I, the outflow of the Cueva del Azufre, and El Azufre II, a tributary of the former. El Azufre I is entirely sulfidic with H<sub>2</sub>S concentrations ranging between 10 and 41 µM/l (Tobler et al. 2006), whereas El Azufre II is fed by several independent sulfidic and non-sulfidic surface springs, which—similarly to cave chamber X—leads to a patchy distribution of H<sub>2</sub>S concentrations from 0 to 150 µM/l (M. Tobler, unpubl. data). Moreover, two adjacent non-sulfidic surface habitats were sampled (Arroyo Cristal, Arroyo Tres; Tobler et al. 2006). All creeks studied eventually drain into the Río Oxolotan, which is part of the Río Grijalva drainage system. A map of the study area and details about the study sites can be found in Tobler et al. (2006).

Fish were collected using seines or dip nets, depending on the habitat type. Data for the two non-sulfidic reference habitats were combined, because few males were caught in Arroyo Cristal ( $n = 5$ ) and Arroyo Tres ( $n = 4$ ), and there was no significant difference in sperm counts between the populations (Mann–Whitney  $U$ -test;  $z = -0.245$ ,  $P = 0.806$ ).

### Laboratory experiment

Both male and female *P. mexicana* from the Río Oxolotan and cave chamber XIII of the Cueva del Azufre were collected in August 2004 and housed in

mixed-sex stock tanks at the University of Oklahoma Aquatic Research Facility, Norman, OK, USA. The mollies used in this experiment were haphazardly chosen individuals from the stock populations, transferred to aquaria (75 l), and allowed to acclimate for 48 h. Fish tanks were equipped with a thin layer of gravel and a filter. A 12:12 h artificial light:dark cycle was maintained.

Fish of the different populations were always kept in separate tanks, which were randomly assigned to either a high or low food treatment, and there was triple replication of the whole  $2 \times 2$  factorial design. Fish were under the respective food treatments for 2 weeks prior to data collection. Four to five males were placed in each aquarium along with two females. Females were included in the study to stimulate sperm production in males (Bozynski and Liley 2003; Aspbury and Gabor 2004). Females were placed in plastic mesh cylinders that allowed visual and chemical stimuli to pass, but physical interactions were not possible, and thus male sperm counts were not affected by potential copulations. Males in the high food treatment were fed one TetraMin® Tropical Tablet daily, whereas males in the low food treatment were fed every second day. Females were fed daily with TetraMin® Tropical Flakes. The floating flakes were too large to pass through the mesh cylinders and were consumed almost immediately and entirely by the females. On the rare occasions that the females did not eat all of their food, excess was removed to prevent any transfer to the males' holding area.

### Semen extraction and sperm counts

Semen was extracted using the same method for both the field and laboratory males. Males were anaesthetized using MS222, rinsed with fresh water, and placed on a wet paper towel. The male's gonopodium was swung forward three times to stimulate sperm release. Then, the body sides were gently massaged from dorsal to the base of the gonopodium using a pair of forceps. Sperm was removed at the base of the gonopodium and transferred into a tube with 100 µl 0.9 M NaCl solution using a micropipette. Because very small amounts of sperm are not easily visible, the base of the gonopodium was rinsed with a drop of the NaCl

solution and this was transferred into the tube as well (Schlupp and Plath 2005). Two drops of the sperm containing solution were transferred to a *Neubauer improved* counting chamber and covered with a slide. Spermatozoa touching the upper and right line of a field were counted for the sample; those touching the lower and left line were omitted. Spermatozoa were quantified in six haphazardly chosen fields and the mean from all six fields was calculated. From the resulting data for the known volume of the counting chamber, the total number of sperm suspended in the aliquot was calculated.

#### Body condition and testes weight

After sperm extraction, the standard length (SL) of each fish was measured to the closest mm and body mass was measured to the closest  $10^{-2}$  g. Condition factor ( $K$ ) was calculated as  $\text{mass}/\text{SL}^3$ . For males from the field, we asked whether low sperm counts would be reflected by a reduction in testes weight in the populations exposed to  $\text{H}_2\text{S}$ . To do so, males collected in the field were stripped of sperm, sacrificed and dissected, and their testes weight was determined. We failed to dissect one male; hence, sample sizes differ between analyses. Laboratory males were revived in heavily aerated fresh water and later returned to stock tanks.

#### Statistical analysis

For the field study, data on sperm counts were log-transformed to achieve normality and analyzed using ANCOVA, in which “population” was a between factor and SL was the covariate. Testes weight data were analyzed using ANCOVA where “population” was a between factor and SL was the covariate. The parametric assumptions of normal distribution and equal variances were met. LSD post-hoc tests were used to compare populations. All  $P$ -values were two-tailed.

For the laboratory study, data on male sperm counts were square root-transformed to achieve normality. Statistical analyses were conducted using ANCOVA, in which “population” and “treatment” were between factors and SL was the covariate. All  $P$ -values were two-tailed.

## Results

### Field study

We detected pronounced differences in the number of sperm cells stripped per male among the five populations examined (ANCOVA:  $F_{4,49} = 14.43$ ,  $P < 0.0001$ ; Tables 1a, 2a; Fig. 1). The post-hoc pair-wise comparison revealed that the males from the non-sulfidic surface habitat as well as the El Azufre II males had more sperm than those from El Azufre I and from both cave chambers (LSD post-hoc test:  $P \leq 0.028$  in all cases). Cave chamber X significantly differed in sperm counts from all other sites (LSD post-hoc test:  $P \leq 0.048$ ) and males from El Azufre I and cave chamber V had significantly less sperm than all other populations (LSD post-hoc test:  $P \leq 0.048$ ). All other post-hoc comparisons were non-significant ( $P \geq 0.066$ ). No effect of SL was detected ( $F_{1,49} = 1.36$ ,  $P = 0.250$ ).

There was a significant difference in the testes weight between males from different populations ( $F_{4,48} = 6.63$ ,  $P < 0.0001$ ; Tables 1a, 2b). The post-hoc test revealed that the males from the non-sulfidic surface habitat had heavier testes than males from the other four populations (LSD post-hoc test:  $P \leq 0.019$ ), whereas gonads of males from cave chamber V were lighter than those of males from all other populations tested (LSD test:  $P \leq 0.022$ ). All other pair-wise comparisons were not significant ( $P > 0.119$ ). Gonad weight positively correlated with body length ( $F_{1,48} = 24.25$ ,  $P < 0.0001$ ). Overall, the weight of males' testes was positively correlated with the amount of sperm stripped per male (Spearman rank-correlation:  $r_s = 0.55$ ,  $P < 0.0001$ ,  $n = 54$ ).

### Laboratory experiments

A significant interaction effect between populations and treatments was detected (ANCOVA:  $F_{1,33} = 4.56$ ,  $P = 0.040$ ), suggesting that sperm production in cave mollies and surface mollies responded differently under food deprivation. Whereas there was no difference in the amount of sperm stripped in the cave population, there was a strong trend for reduced sperm availability under the low food treatment in the surface population (Tables 1b, 2c; Fig. 2). There was

**Table 1** Standard length, body weight, testes weight, and condition in the five populations studied

Population	Habitat/treatment	<i>n</i>	SL (mm)	Body weight (g)	Testes weight (g)	<i>K</i>
<i>(a) Field data</i>						
Arroyo Cristal & Arroyo Tres	L, N	9	38.3 ± 13.0	1.616 ± 1.578	0.018 ± 0.008	0.022 ± 0.003
El Azufre II	L, M	10	30.1 ± 3.9	0.596 ± 0.209	0.007 ± 0.005	0.021 ± 0.002
El Azufre I	L, S	13	30.2 ± 4.7	0.543 ± 0.171	0.007 ± 0.006	0.020 ± 0.005
Chamber V	D, S	12	29.4 ± 3.1	0.428 ± 0.071	0.001 ± 0.003	0.017 ± 0.003
Chamber X	D, S	11	32.8 ± 1.7	0.598 ± 0.127	0.011 ± 0.007	0.017 ± 0.002
<i>(b) Laboratory data</i>						
Cave	LF	8	30.9 ± 3.0	0.656 ± 0.261		0.021 ± 0.002
Cave	HF	7	31.3 ± 2.4	0.700 ± 0.140		0.023 ± 0.002
Surface	LF	12	28.0 ± 4.6	0.464 ± 0.204		0.020 ± 0.004
Surface	HF	11	28.1 ± 6.4	0.564 ± 0.572		0.021 ± 0.002

*n*, Number of individuals; SL, standard length; *K*, condition factor. Habitat variables are defined as follows: L, light; D, dark; N, non-sulfidic; M, mixed (sulfur/non-sulfidic); S, sulfidic. Treatment variables are defined as LF, low food treatment; HF, high food treatment. Data are given as mean ± SD

**Table 2** Analysis of covariance on sperm count and testes weight as influenced by population identity

Source	df	Mean square	<i>F</i>	<i>P</i>
<i>(a) Field data: sperm count</i>				
SL	1	0.990	1.356	0.250
Population	4	10.537	14.432	<b>&lt;0.0001</b>
Error	49			
<i>(b) Field data: testes weight</i>				
SL	1	0.001	24.247	<b>&lt;0.0001</b>
Population	4	0.000	6.247	<b>&lt;0.0001</b>
Error	48			
<i>(c) Laboratory data: sperm count</i>				
SL	1	2.49E+13	1.644	0.209
Population	1	1.40E+13	0.928	0.342
Treatment	1	3.92E+12	0.259	0.614
Population * Treatment	1	6.89E+13	4.560	<b>0.040</b>
Error	33			

Standard length (SL) was used as a covariate. Significant values are in bold font

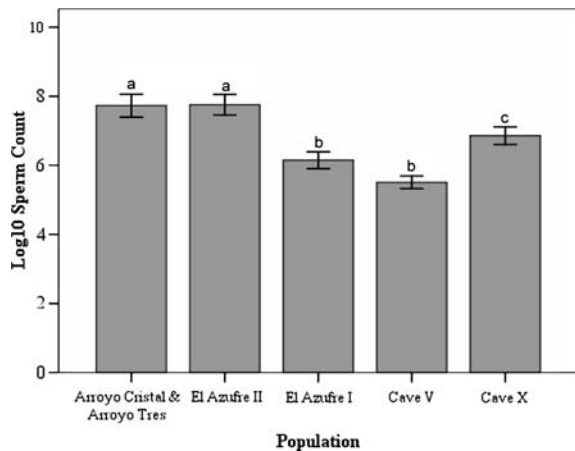
no effect of SL ( $F_{1,33} = 1.64$ ,  $P = 0.209$ ) on the amount of sperm stripped.

## Discussion

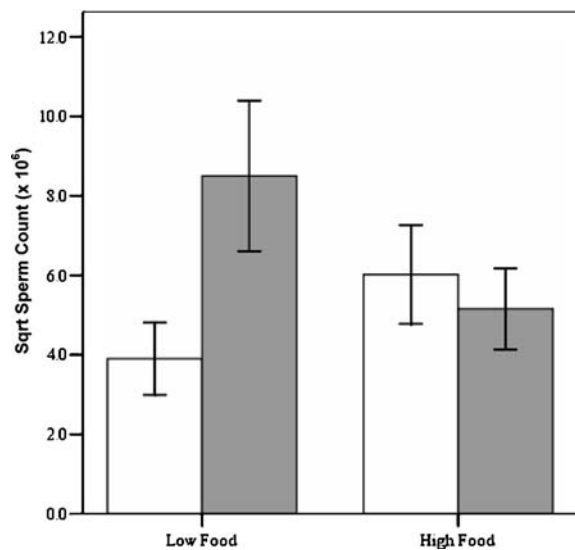
Male *P. mexicana* inhabiting constantly sulfidic habitats had the least amount of strippable sperm, whereas their conspecifics living in habitats with no H<sub>2</sub>S present had the most. In the two habitats in

which fish are able to move between sulfidic and non-sulfidic patches within short distances, males had intermediate amounts of sperm irrespective of being from a surface or cave dwelling population. Thus, the toxicity of H<sub>2</sub>S likely has a higher impact on sperm production than the absence of light.

H<sub>2</sub>S may affect sperm production in that it diverts energy to behavioral or physiological processes necessary to cope with the toxicant. Among other things, high concentrations of H<sub>2</sub>S inhibit electron



**Fig. 1** Analysis of covariance showed a significant difference in sperm counts ( $\pm$ SE) among populations of *P. mexicana* ( $F_{4,49} = 14.432$ ,  $P < 0.0001$ ). Pair-wise post-hoc tests (LSD,  $\alpha < 0.05$ ) revealed which populations differed as labeled by superscripts



**Fig. 2** Analysis of covariance detected a significant interaction effect between populations and treatments in sperm counts ( $\pm$ SE) among populations of *P. mexicana* ( $F_{1,33} = 4.560$ ,  $P = 0.040$ ). Gray bars represent the cave population, whereas white bars represent the surface population

transport in aerobic respiration and thus are acutely toxic for most eukaryotic organisms (Evans 1967; Theede 1973; Nicholls 1975; Smith et al. 1977; Carrico et al. 1978; Stallones et al. 1979; Bagarinao 1992; Grieshaber and Völkel 1998). Some fishes are able to detoxify sulfide to some extent (Bagarinao

1992); e.g., they are capable of oxidizing sulfide to thiosulfate in liver mitochondria (Bagarinao and Vetter 1990) or binding sulfide to ferrous and ferric hemoglobin and other blood compounds (Evans 1967; Bagarinao 1992; Bagarinao and Vetter 1989). Although it is yet unclear how *P. mexicana* residing in sulfide-rich habitats cope with the chronic exposure to  $H_2S$ , short-term survival seems to be dependent on energy availability (Plath et al. in press). Thus, having to cope with a physiological stressor may reduce the allotment of energy available for other life history needs, such as sperm production. This is also reflected on a morphological level, since males from sulfidic habitats also had reduced testes weights.

In line with the reduction of male sperm production, energetically demanding behaviors like aggressive and sexual behaviors were shown to be dramatically reduced in the cave and the El Azufre I (Parzefall 2001; Plath et al. 2003), potentially as an adaptation to save energy under the extreme environmental conditions. Furthermore, reduced sperm counts and reduced male sexual behavior were also documented in fish inhabiting waters polluted by anthropogenic influence (Toft and Guillette 2005). Since the reduced sexual activity can also be found in El Azufre I males and cave molly males that had been reared in the absence of  $H_2S$ , the reduced male sexual behavior seems to have a genetic basis in these populations (Plath et al. 2003, 2006b).

The laboratory experiment revealed that cave molly males are capable of producing similar amounts of sperm as males from the surface population. This was not only true in the high food treatment, but cave mollies produced even more sperm when energy availability was reduced. Most likely, the conditions provided in the low food experimental treatment were less harsh than the conditions cave mollies encounter in their natural habitat. The fact that there was a significant interaction between population and food treatment, with cave mollies producing more sperm under low food, may hint towards a higher starvation resistance in the cave population.

Invertebrates as well as vertebrates of hypogean habitats are known to have lower energetic requirements than surface-dwelling relatives. These adaptations allow individuals to survive longer periods of food deprivation (Poulson 1963; Hüppop 2000;

Hervant et al. 2001). Although the Cueva del Azufre may be considered nutrient-rich compared to many other karst systems because of its chemoautotrophic production and the input of bat guano (Langecker et al. 1996), its residents suffer from low body condition (Tobler et al. 2006). Like other hypogean or extremophile species, cave mollies may have evolved lower metabolic rates which allow for survival and reproduction in times of high physiological stress.

The data from the present study support the idea that the cave molly is one of the few extremophile vertebrates (sensu Townsend et al. 2003): extreme environmental conditions appear to require energetically costly adaptations in this system, and males allocate less energy into gonad development and ultimately sperm production when residing in H<sub>2</sub>S-rich habitats.

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