Reduction of Energetic Demands through Modification of Body Size and Routine Metabolic Rates in Extremophile Fish

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ABSTRACT

Variation in energy availability or maintenance costs in extreme environments can exert selection for efficient energy use, and reductions in organismal energy demand can be achieved in two ways: reducing body mass or metabolic suppression. Whether long-term exposure to extreme environmental conditions drives adaptive shifts in body mass or metabolic rates remains an open question. We studied body size variation and variation in routine metabolic rates in locally adapted populations of extremophile fish (*Poecilia mexicana*) living in toxic, hydrogen sulfide–rich springs and caves. We quantified size distributions and routine metabolic rates in wild-caught individuals from four habitat types. Compared with ancestral populations in nonsulfidic surface habitats, extremophile populations were characterized by significant reductions in body size. Despite elevated metabolic rates in cave fish, the body size reduction precipitated in significantly reduced energy demands in all extremophile populations. Laboratory experiments on common garden–raised fish indicated that elevated routine metabolic rates in cave fish likely have a genetic basis. The results of this study indicate that adaptation to extreme environments directly impacts energy metabolism, with fish living in cave and sulfide spring environments expending less energy overall during routine metabolism.

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Introduction

Animals require energy for maintenance, growth, and reproduction, and since individuals' energy expenditure may be greater or less than the environmental energy availability, they can modulate a variety of physiological processes to balance energy supply and expenditure (Cho et al. 1982). Metabolic rate is a physiological measure of the rate at which organisms burn calories from assimilated food resources to produce energy for organismal functioning, and understanding metabolic rate variation is critical for investigating ecological processes at multiple levels of organization (Brown et al. 2004; Sibly et al. 2012). The majority of metabolic rate variation in animals coincides with variation in body mass and temperature (Peters 1983; Gillooly et al. 2001; Brown et al. 2004; Clarke and Fraser 2004; Cano and Nicieza 2006). Nonetheless, mass- and temperature-adjusted metabolic rates can vary substantially even among closely related taxa (McNab 1986; Clarke and Johnston 1999; Nagy et al. 1999; Lovegrove 2000; Schaefer and Walters 2010). Elucidating the selective forces that shape such residual metabolic rate variation in allometric plots and underlie macroevolutionary patterns in the diversification of metabolic rates is a critical challenge in physiology (Garland and Carter 1994; Feder et al. 2000).

Resource availability is a strong source of selection driving adaptive modification of metabolic rates within and among closely related species (Mueller and Diamond 2001; McCue 2010; Moiroux et al. 2012). From an energetic point of view, fitness can be described as the conversion rate of energy into offspring (Brown et al. 1993), which ultimately is limited first by the rate at which organisms can acquire energy from the environment and then by the rate at which they can allocate energy to reproduction (as opposed to maintenance or growth). Consequently, reductions in environmental resource availability or increases in maintenance or growth costs are predicted to constrain the amount of energy for reproduction and exert selection for a reduced overall energy demand that allows for maximizing relative energy allocation to the production of offspring. Organisms can reduce their overall energy demand in two fundamental ways. First, they can reduce their body size by reducing energy allocation to growth, which simultaneously reduces total energy expenditure for maintenance

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(Blanckenhorn 2000; Wikelski and Romero 2003; Pafilis et al. 2009; McNab 2010). Second, organisms can reduce metabolic rate independently of body size, which changes the allometric relationship between metabolism and body size (Guppy and Withers 1999; Wang et al. 2006; Burton et al. 2011). It is important to note that these mechanisms are not mutually exclusive but may work in synchrony, such that focusing merely on body size or metabolic rates alone can lead to erroneous conclusions (McNab 1999, 2002; Van Voorhies et al. 2004; McCue 2010).

Systems in which closely related populations occur in habitats with starkly different environmental conditions provide an excellent opportunity to study evolutionary change in organismal energy demand. This is especially true for species that have invaded extreme environments, and comparisons between populations in localized extreme environments and adjacent "benign" habitats allow for a powerful approach to examine the effects of stressors on organismal physiology as well as the evolutionary trajectories of populations. Exposure to physicochemical stressors profoundly affects energy budgets of organisms because the maintenance of homeostasis precipitates in considerable energetic costs through investments in physiological, morphological, or behavioral coping mechanisms (Calow 1989; Sibly and Calow 1989; Parsons 1996). As such, continuous exposure to environmental stressors should select for increased metabolic rates (e.g., Knoblauch et al. 1999). However, the evolution of increased metabolic rates in extreme environments is typically constrained by a reduced supply of resources required for metabolic expenditure (Waterman 1999, 2001). While many studies have investigated immediate metabolic costs of exposure to physicochemical stressors and energy limitation (e.g., Haney and Nordlie 1997; Penttinen and Kukkonen 1998; Rose et al. 2006; Wang et al. 2006; McKenzie et al. 2007; McCue 2010), it remains unclear how metabolic rates and metabolic rate plasticity evolve when populations adapt to diverse environmental stressors. Here, we explicitly tested for differences in body size and routine metabolic rate in the context of resource limitation in extremophile fish. Such information will be important for a more general understanding of metabolic rate evolution (Naya et al. 2013).

Poecilia mexicana is a livebearing fish that has repeatedly colonized extreme environments characterized by the presence of naturally occurring toxic hydrogen sulfide (H_2S) and the absence of light in caves (Tobler and Plath 2011; Tobler et al. 2011). In Mexico's Cueva del Azufre system, both environmental factors occur in a natural, factorial design forming four distinct habitat types: nonsulfidic surface streams, sulfidic surface streams, a nonsulfidic cave, and a sulfidic cave (Tobler et al. 2008*a*; Plath and Tobler 2010). Populations from different habitat types are characterized by adaptive trait divergence (Tobler et al. 2008*a*; Tobler and Plath 2011) and are reproductively isolated with low rates of gene flow among populations living under different environmental conditions (Plath et al. 2007*a*, 2010). Reproductive isolation is mediated at least in part by natural selection against nonadapted, migrant individuals and by sexual selection through ecotype-assortative mating (Tobler 2009; Tobler et al. 2009*b*; Riesch et al. 2011*a*).

The strikingly different environmental conditions in this system are expected to profoundly affect organismal energy budgets. Caves have been widely considered as energy limited because of a lack of photosynthetic primary production (Poulson and White 1969; Langecker 2000), and some cave organisms accordingly have evolved lower metabolic rates (e.g., Hüppop 1985, 1986; Hervant et al. 2000; Poulson 2001; Wilhelm et al. 2006). Similarly, exposure to H_2S has been shown to constrain energy acquisition in *P. mexicana* (Tobler et al. 2009*a*). H_2S causes and aggravates hypoxia in natural environments (Bagarinao 1992), driving exposed fish to trade off time between benthic foraging and aquatic surface respiration, which directly mediates survival in the toxic and hypoxic environment (Plath et al. 2007*b*; Tobler et al. 2009*a*). In addition, exposure to perpetually sulfidic environments requires active detoxification for survival. Sulfide spring fish exhibit a heritable and constitutive increase in H_2S detoxification ability through upregulation of the sulfide:quinone oxidoreductase pathway (Tobler et al. 2014), a process that is energetically costly (Ip et al. 2004; Hildebrandt and Grieshaber 2008). Accordingly, some organisms adapted to sulfidic environments have been documented to increase energy consumption in the presence of H_2S (Gorodezky and Childress 1994; Schneider 1996). Clearly, exposure to extreme environmental conditions impacts organismal energy budgets through reduced energy availability, reduced ability for energy acquisition, and/or increased organismal maintenance costs, which is reflected in *P. mexicana* from both sulfidic and cave environments consistently having a lower body condition than fish from nonsulfidic surface habitats (assessed through abdominal distension [Plath et al. 2005], body fat content [Tobler 2008], and mass-length relationships [Tobler et al. 2006]). Consequently, adaptation to these environments should be linked to energy metabolism, and we hypothesized that extremophile populations should be selected for reductions in overall energy demand. To test this overarching hypothesis, we addressed the following objectives: (1) we quantified size distributions of fish in different habitat types to test whether adaptation to extreme environments was associated with body size reduction; (2) we quantified routine metabolic rates in wild-caught individuals to test whether adaptation to extreme environments was associated with metabolic rate suppression; and (3) we tested for a genetic basis of variation in metabolic rates and metabolic rate plasticity in response to energy availability by quantifying routine metabolic rates in common garden–raised individuals subjected to different food treatments.

Material and Methods

Study Sites

To disentangle potential effects of the presence of hydrogen sulfide and permanent darkness in caves on organismal energy demands, we focused on a set of habitats in the Cueva del Azufre system, where these environmental factors occur in a

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natural, factorial design: (1) a nonsulfidic surface habitat (Arroyo Bonita), (2) a sulfidic surface habitat (El Azufre), (3) a nonsulfidic cave (Cueva Luna Azufre), and (4) a sulfidic cave (Cueva del Azufre, chamber V). All sites were located within 4 km of each other and were situated near the village of Tapijulapa in the Mexican state of Tabasco (Tobler et al. 2008*a*). The sulfidic cave is segregated into different chambers with varying exposure of light and high densities of *Poecilia mexicana* (Gordon and Rosen 1962; Parzefall 2001), while the nonsulfidic cave is considerably smaller than the sulfur cave, completely dark, and maintains only a small *P. mexicana* population (Tobler et al. 2008*b*). Fish from the sulfidic surface habitat were collected in the El Azufre, a stream that drains the Cueva del Azufre and eventually joins the Rio Oxolotan. The nonsulfidic habitat (Arroyo Bonita) is also a tributary of the Rio Oxolotan and is similar in size and structure to that of the El Azufre (Tobler et al. 2008*a*). All procedures conducted for this study were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Animal Care and Use Protocol AS10-15).

Size Distribution in Natural Populations

To compare size distributions in the four habitat types, we assembled data from previous studies (Tobler 2008; Tobler et al. 2006, 2008*a*, 2008*b*, 2008*c*) as well as several unpublished projects. In all cases, fish were collected using seines (4 m long, 4 mm mesh width), sexed, and weighed (blotted wet weight to the closest 0.01 g). Mass-based size distributions were then analyzed using ANOVA with body mass as a dependent variable. We included sex, presence or absence of H_2S (i.e., sulfidic vs. nonsulfidic habitat), and presence or absence of light (i.e., cave vs. surface habitat) as independent variables.

Determining Routine Metabolic Rates in Natural Populations

For the quantification of metabolic rates in wild-caught fish, specimens were collected in June 2012. On capture, fish were immediately transferred into insulated coolers with aerated water and transported to a nearby field station at the Centro de Investigación e Innovación para la Enseñanza y el Aprendizaje in Teapa, Tabasco. Before metabolic rate trials, fish were allowed to acclimate to laboratory conditions for at least 48 h. During that time, they were kept in 70-L tanks with filtered and aerated water. The temperature was kept between 24° and 26°C. All fish were subjected to a 12L : 12D photoperiod.

We employed a closed-chamber respirometry approach to quantify an individual's routine metabolic rate, which is defined as the oxygen consumption of unconstrained, postabsorptive organisms capable of spontaneous motor activity (Fry 1957; Steffensen 1989). This approach has been widely used to quantify metabolic costs associated with a variety of factors, including exposure to environmental stressors (Haney and Nordlie 1997; Pirozzi and Booth 2009), locomotion (Basolo and Alcaraz 2003; Seibel and Drazen 2007), elaborate morphological structures (Allen and Levinton 2007), mating behaviors (Hoback and Wagner 1997), and gestation (Timmerman and Chapman 2003). The protocol for measurements of oxygen consumption included the following steps for each individual. (1) As detritivores, *P. mexicana* have a relatively fast gut passage time of <6 h (M. Tobler and K. Scharnweber, unpublished data). Hence, fish were not fed 24 h before trials to ensure that metabolic rate measurements were conducted on postabsorptive individuals (Timmerman and Chapman 2004*b*; Norin and Malte 2011). (2) Fish were haphazardly chosen from stock tanks and placed into individual respirometry bottles filled three-fourths with water for a 12-h acclimation period under continuous aeration. Bottles had a total volume of 580 mL and were painted solid black to prevent light penetration. Four bottles were placed together in a black equipment box with a lid to further minimize light exposure and with water to minimize temperature fluctuations in the respirometry bottles. Realized mean temperatures in respirometry bottles ranged from 26.1° to 27.2°C across all trials. (3) After the acclimation period, the respirometry chambers were flushed with fresh, aerated water to remove metabolic waste products (Timmerman and Chapman 2004*a*) and capped with a fitted lid that allowed for the insertion of an oxygen probe. Once capped, water was added to the chamber using a squirt bottle to remove any excess air, and a YSI ProDO optical dissolved oxygen probe (YSI, Yellow Springs, OH) was inserted into each bottle (this probe monitors dissolved oxygen concentration in conjunction with temperature). Plumbers putty was fitted around the oxygen probe to prevent any diffusion of gases. Once all four respirometry bottles were set up, the lid of the water bath was closed, and the probes were set to measure the dissolved oxygen concentration at 10-s intervals. All experiments were run for at least 6 h or until the oxygen saturation reached 10%, to prevent mortality. Probes were recalibrated regularly according to the manufacturer's recommendations to maintain accuracy. Note that all metabolic rate trials were conducted in the absence of H_2S, even for sulfidic populations, because the reactivity of H_2S with oxygen in aqueous solution (Chen and Morris 1972) affects estimates of organismal oxygen consumption. (4) After termination of a trial, individuals were weighed (blotted wet weight to the closest 0.01 g) and sexed. Descriptive statistics for the body mass of individuals used are given in table 1.

Raw data obtained from all trials represented measurements of oxygen concentration and temperature through time. For each individual trial, we first removed any outliers that were likely caused by instrumental error (<0.1% of data points). We also removed any data points from the first 60 min of each trial, as the flushing of the respirometry bottle with fresh water and the installation of the oxygen probe may have caused erratic fish activity (Timmerman and Chapman 2004*b*). Because fish metabolic rates may be affected by reduced ambient oxygen concentrations (Haney and Nordlie 1997; Ultsch et al. 1978), we included only data points measured at dissolved oxygen saturations >70%. Metabolic rate (mg O_2/h) was then calculated for each individual as the slope of a regression (multiplied by the volume of water in the respiratory bottle) with

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Table 1: Descriptive statistics for body masses as well as sample sizes of fish used in metabolic rate trials with wild-caught and common garden–raised individuals of *Poecilia mexicana*

			Laboratory-raised females		Laboratory-raised males	
	Wild-caught females	Wild-caught males	High food	Low food	High food	Low food
Nonsulfidic surface:						
Mean ± SD	1.07 ± .47	1.07 ± .80	1.01 ± .33	1.09 ± .37	.70 ± .17	.52 ± .04
Range	.23–1.88	.26–2.32	.39–1.67	.61–1.69	.52–.91	.48–.58
N	15	5	23	24	6	4
Sulfidic surface:						
Mean ± SD	1.04 ± .45	.74 ± .21	1.17 ± .53	.49 ± .10	.54 ± .09	.48 ± .08
Range	.43–2.04	.51–.98	.56–2.52	.34–.66	.41–.71	.36–.67
N	16	4	11	10	17	19
Nonsulfidic cave:						
Mean ± SD	.58 ± .21	.24 ± .08	1.08 ± .27	.94 ± .35	.53 ± .11	.54 ± .14
Range	.21–.81	.16–.41	.53–1.88	.73–1.81	.41–.74	.38–.82
N	7	8	21	15	8	13
Sulfidic cave:						
Mean ± SD	1.15 ± .36	.69 ± .55	1.02 ± .50	.91 ± .42	.69 ± .13	.58 ± .16
Range	.36–1.71	.40–2.00	.55–2.03	.50–1.66	.45–.82	.29–.45
N	12	7	15	11	12	13

Note. All measurements of body mass are provided as mean ± SD and range (minimum to maximum) in grams; the sample size (N) of each experimental group is also provided. Overall sample size was $N = 74$ for the field experiment and $N = 222$ for the laboratory experiment.

oxygen concentration as a dependent variable and time as an independent variable. For all regressions, R^2 was >0.90.

Routine metabolic rate data were analyzed using ANCOVA. Log_{10}-transformed routine metabolic rate was used as a dependent variable. We included sex, presence or absence of H_2S in natural populations (i.e., sulfidic vs. nonsulfidic habitat), and presence or absence of light in natural populations (i.e., cave vs. surface habitat) as independent variables. Temperature and mass ($\log_{10}$ transformed) were included as covariates in all models. All three-way ($F \leq 1.909$, $P \geq 0.173$) and two-way ($F \leq 2.780$, $P \geq 0.101$) interaction terms were not significant and thus excluded from the final model.

Genetic Basis of Variation in Routine Metabolic Rates and Metabolic Rate Plasticity

Metabolic rate variation in wild-caught fish may merely reflect plastic responses to life under different environmental conditions. Hence, we tested whether differences in routine metabolic rates documented in wild-caught individuals have a genetic basis by using common garden–raised fish from the same populations investigated in the field. In addition, we used experimental manipulations of energy availability to test for population differences in metabolic rate plasticity in response to energy availability.

Common garden–raised fish came from stocks at the University of Oklahoma and Oklahoma State University. All animals used in the laboratory portion of this study were born and raised in captivity, and fish were maintained under nonsulfidic conditions with a 12L∶12D photoperiod. Individuals for the metabolic rate experiment were haphazardly chosen from stock tanks. Each fish was sexed and weighed (blotted wet weight to the closest 0.01 g; table 1), and five fish from the same population were introduced into a 40-L tank with filtered and aerated water (these are low stocking densities compared with regular stock tanks and were used to minimize competition between individuals). Tanks were assigned to different food treatments in a balanced fashion (i.e., neighboring tanks alternated in population and food treatment assignment) for a total of eight tanks per treatment. During the experiment, all fish were fed with Earthworm Fish Flakes (American Brine Shrimp Company, Ogden, UT). To standardize resource availability, we calculated the total fish mass for each tank. For the high food treatment, we calculated the amount of food (HF, in grams) as $\text{HF} = 0.0125 \times (\text{total fish mass})^{0.65}$. Low food treatment groups received half the amount of food provided to high food treatment groups. Fish were fed the calculated amount of food twice a day from Monday through Friday and once a day during the weekend. All fish were kept on their respective food treatment for at least 21 d before testing. During this time, the temperature was kept constant at 25°C. Upon completion of a trial, fish were returned to regular stock tanks.

In general, the experimental protocol for metabolic rate measurements was identical for the wild-caught and common garden–raised fish. There were only two critical differences. First, instead of handheld oxygen probes, the oxygen consumption measurements were conducted using a Loligo Systems (Tjele, Denmark) four-channel respirometry system with fiber optic probes. Oxygen concentrations and temperature were measured every second. Second, the temperature in the water bath was controlled using an Ebo-Jäger 75-W aquarium

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heater (EHEIM, Deizisau, Germany) in conjunction with a Mighty Pro chiller (AquaEuroUSA, Gardena, CA). Fish were tested at 20°, 25°, or 30°C, to test for potential population differences in the temperature dependence of metabolic rates. The four habitat types investigated here vary in both mean temperatures and temperature variability (with extreme environments typically exhibiting higher averages and lower variability), and the chosen temperatures reflect the range that *P. mexicana* typically encounter in natural habitats (Tobler et al. 2006, 2008*a*).

Statistical analyses were conducted as outlined above for the wild-caught fish, except that the ANCOVA model for the laboratory-reared fish also included food treatment (high vs. low) as an independent variable. Three-way interactions ($F \leq 2.684$, $P \geq 0.103$) as well as interaction terms including temperature ($F \leq 0.379$, $P \geq 0.539$) were not significant and hence excluded from the final model.

Results

Size Distributions

Overall, we assembled mass data for 1,454 individuals. ANOVA indicated that males exhibited a significantly smaller body size than females in all populations (table 2, pt. A), which is likely related to the fact that male poeciliids, unlike females, have determinate growth (Constantz 1989; Reznick and Miles 1989). More importantly, we detected a significant cave × sulfide interaction (as well as significant cave and sulfide main effects), indicating significant body size differences among populations residing in different habitat types. Individuals from the ancestral population in the nonsulfidic surface habitat were by far the largest, with a mass of 2.01 ± 1.20 g (mean ± SD; fig. 1*A*). In contrast, all individuals from extreme environments exhibited a reduction in overall body mass, with individuals >3.00 g being exceedingly rare (fig. 1*B*–1*D*). Among

Table 2: Results of ANOVA and ANCOVA explaining variation in body size and metabolic rates

Variable	df	F	P	η_p^2
A. Body mass ($\log_{10}$ transformed):				
Sex	1	149.682	<.001	.100
Cave	1	398.866	<.001	.228
Sulfide	1	100.839	<.001	.070
Sex × cave	1	.660	.417	<.001
Sex × sulfide	1	1.632	.202	.001
Cave × sulfide	1	292.731	<.001	.178
B. Routine metabolic rate of wild-caught fish ($\log_{10}$ transformed):				
Sex	1	.001	.971	<.001
Cave	1	9.458	.003	.122
Sulfide	1	3.322	.073	.047
Log(mass)	1	210.822	<.001	.756
Temperature	1	.884	.351	.013
C. Routine metabolic rate of laboratory-reared fish ($\log_{10}$ transformed):				
Sex	1	.019	.889	<.001
Food	1	31.87	<.001	.137
Cave	1	7.616	.006	.037
Sulfide	1	.294	.589	.001
Log(mass)	1	76.603	<.001	.277
Temperature	1	265.84	<.001	.571
Food × cave	1	1.484	.225	.007
Food × log(mass)	1	10.438	.001	.05
Food × sex	1	3.859	.051	.019
Food × sulfide	1	3.34	.069	.016
Cave × log(mass)	1	1.315	.253	.007
Cave × sex	1	.575	.449	.003
Cave × sulfide	1	.007	.933	<.001
Sex × log(mass)	1	.257	.613	.001
Sulfide × log(mass)	1	.161	.689	.001
Sulfide × sex	1	.082	.775	<.001

Note. Part A shows comparison of size distributions of fish residing in different habitat types, part B shows comparison of routine metabolic rates in wild-caught individuals, and part C shows comparison of routine metabolic rates in common garden–raised individuals subjected to different resource availability treatments. Note that the effect size for each of the terms in a model was estimated by use of partial eta squared (η_p^2).

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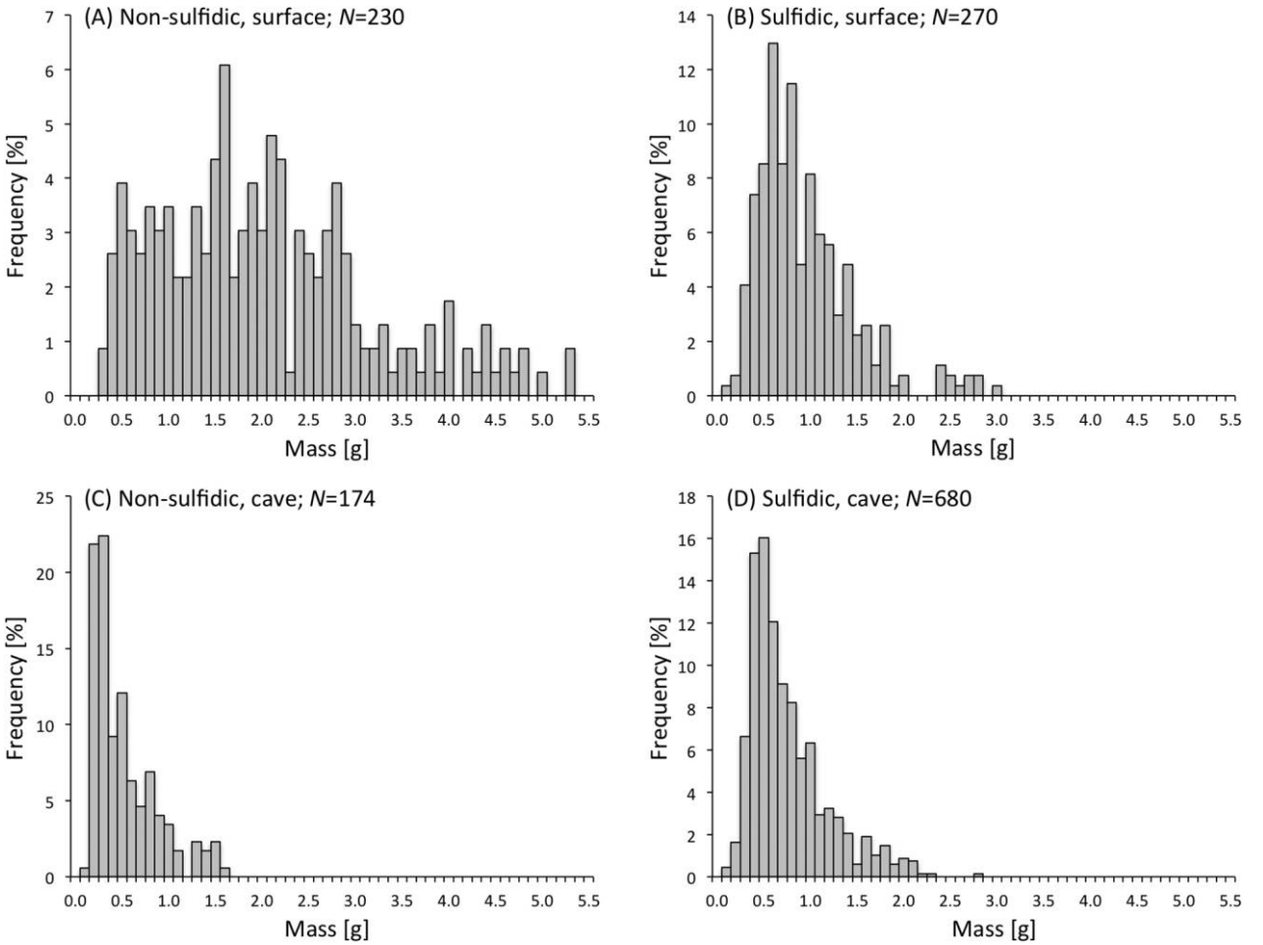

Figure 1. Frequency distributions based on body mass in natural populations. *A*, Arroyo Bonita; *B*, El Azufre; *C*, Cueva Luna Azufre; *D*, Cueva del Azufre. Frequencies are expressed as the percentage of individuals within a size class in each population.

the populations inhabiting extreme environments, sulfidic surface fish were the largest (0.92 ± 0.53 g), sulfidic cave individuals were intermediate (0.75 ± 0.48 g), and fish from the nonsulfidic cave were the smallest (0.48 ± 0.34 g).

Routine Metabolic Rate Variation in Natural Populations

Analysis of the wild-caught fish revealed significant variation in routine metabolic rates among locally adapted populations. Body mass, as expected, explained most variation in routine metabolism (table 2, pt. B). While there was no difference between sulfidic and nonsulfidic populations, cave fish exhibited significantly higher routine metabolic rates than surface fish (fig. 2*A*). Temperature did not significantly affect metabolic rates in wild-caught fish, likely because the temperature range was relatively narrow (26°–27°C).

Routine Metabolic Rates and Metabolic Rate Plasticity in Common Garden–Raised Fish

Temperature and mass explained the bulk of variation in routine metabolic rates of laboratory-reared individuals (table 2, pt. C; fig. A1; figs. A1–A3 are available online). Furthermore, laboratory experiments confirmed the significantly higher routine metabolic rates in cave populations (fig. 2*B*), as documented in wild-caught fish. Similarly, there was no significant difference between fish from sulfidic and nonsulfidic habitats. In addition, fish receiving the high food treatment exhibited higher routine metabolic rates than those receiving low food treatments, although these differences were dependent on body mass (see the significant food × mass interaction term in table 2, pt. C). Specifically, reductions in routine metabolic rates were more pronounced in larger individuals than in smaller ones (fig. 3). Finally, there was no evidence for population differences in response to food treatments or in the temperature dependence of metabolic rates.

Discussion

Our study revealed significant variation in traits associated with energy metabolism among locally adapted populations of *Poecilia mexicana* inhabiting contrasting environments characterized by the presence or absence of light and toxic hydrogen sulfide. In particular, we found significant reductions in

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body size in extremophile populations and significant among-population variation in routine metabolic rates of wild-caught individuals, although analyses of body size variation and metabolic rates yielded somewhat contradictory results. Laboratory experiments using common garden–raised fish revealed genetic variation in routine metabolic rates, likely indicating evolved differences in energy metabolism among populations of the same species.

Reducing Organismal Energy Demands: Patterns and Mechanisms

Reduced resource availability and increased maintenance costs often associated with extreme environments are predicted to exert selection for reduced energy demands, allowing organisms adapted to the extreme conditions to maximize the relative investment into reproduction (Brown et al. 1993; Parsons 1996). Our analyses indicated significant reductions in body mass for all populations from habitats with extreme environmental conditions, supporting the notion that selection acts to reduce organismal energy demands. Contrary to predictions, however, cave fish exhibited significantly higher routine metabolic rates than fish from surface populations (irrespective of the presence of H_2S in natural waters). These contradictory results beg the question of whether variation in body size and metabolic rate balance each other in a way that overall organismal energy demands do not vary among populations living under different environmental conditions or whether reductions in body mass outweigh increases in routine metabolic rates. Simulating total energy expenditure of average individuals from the different populations under simultaneous consideration of population-specific size distributions and allometric metabolic rate functions (see the appendix, available online, for details) indicated that body mass reductions outweigh differences in routine metabolic rate. In fact, estimates of total energy expenditure were significantly and substantially reduced (between 27% and 49%) in fish from extreme habitats compared with that in the ancestral nonsulfidic surface population (fig. A2), which provides unequivocal evidence for a reduction in energy demands in extreme environments. Hence, the reduction in body size outweighed the increase in metabolic rates, highlighting that variation in body size and metabolic rates need to be considered simultaneously because investigating one without the other may lead to spurious conclusions (e.g., McNab 1999).

Reductions in energy demands in extremophile populations were primarily driven by modification of body size rather than metabolic rate suppression. This parallels results from selection experiments, in which mice selected for high locomotor activity maintained stable energy budgets despite high costs because they exhibited reductions in body size rather than reductions in mass-specific costs associated with running (Rezende et al. 2009). Consequently, modification of body size may face fewer evolutionary constraints than modification of metabolic rates, which would explain the tight correlation between mass and metabolic rate across a broad range of taxa (Gillooly et al. 2001; Brown et al. 2004). While common-garden experiments revealed a genetic component to variation in routine metabolic rates (cave fish retained elevated routine metabolic rates when raised in the laboratory for multiple generations), it remains unclear whether variation in body size among populations living in contrasting environments is driven by genetically based evolutionary change or phenotypic plasticity. Particularly in fishes that have indeterminate growth, there is substantial evidence for both heritable and plastic components underlying variation in body size (e.g., Campton 1992; Hughes et al. 2005; Hard et al. 2008). Overall, our study provides strong evidence that living in and adapting to extreme environments is linked to modification of energy metabolism, even though proximate mechanisms remain to be studied and ultimate mechanisms may differ between sulfidic and cave habitats.

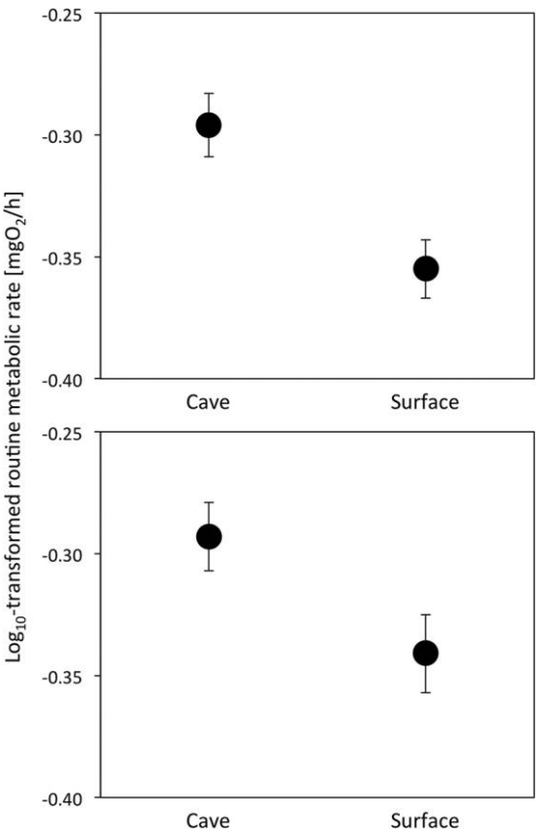

Figure 2. *A*, Differences in routine metabolic rates between cave and surface populations for wild-caught individuals. Depicted are estimated marginal means (±SD) of routine metabolic rates derived from the analytical model in table 2, part B. Mean values of covariates used for the calculation of estimated marginal means were as follows: mass ($\log_{10}$ transformed) = −0.134 g, temperature = 26.7°C. *B*, Differences in routine metabolic rates between cave and surface populations for laboratory-reared individuals. Depicted are estimated marginal means (±SD) of routine metabolic rates derived from the analytical model in table 2, part C. Mean values of covariates used for the calculation of estimated marginal means were as follows: mass ($\log_{10}$ transformed) = −0.125 g, temperature = 25.9°C.

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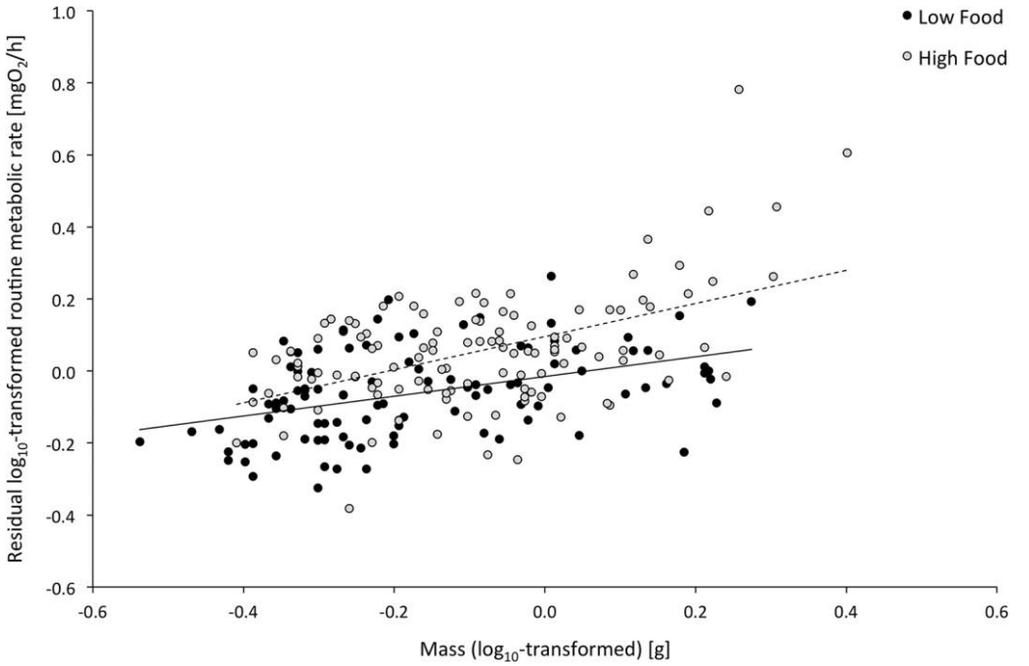

Figure 3. Relationship between body mass and routine metabolic rates for laboratory-raised fish receiving the high and low food treatments. Depicted are residuals from the analytical model (without mass and food treatment) presented in table 2, part C.

Metabolic Rate Variation in Cave Environments

Caves are typically considered to have low resource availability due to the lack of photosynthetic primary production (Poulson and White 1969; Langecker 2000). Accordingly, a diversity of cave organisms have been reported to exhibit reduced metabolic rates compared with close relatives from surface habitats (see Hüppop 1985 for a review). To our knowledge, no previous studies have investigated to what degree body size reduction and metabolic rate suppression have contributed to decreases in energetic demands. However, it is important to note that inferences about energy metabolism in other cave organisms have primarily focused on mass-adjusted routine metabolic rates (e.g., Poulson 1963; Culver and Poulson 1971; Hüppop 1985; Hervant et al. 1997; Hervant et al. 2001), and—at least in some cases—there is evidence for selection for increased body size in cave populations (Culver et al. 1995; Christiansen 2012), presumably to increase starvation resistance in temperate caves with temporal periodicity of food (Hüppop 2000). Contrary to other cave organisms, our data indicate that cavernicolous individuals of *P. mexicana* have higher routine metabolic rates than relatives from surface habitats, such that overall reductions in energetic demands are primarily driven by reductions in body size. This discrepancy may be explained by the fact that resource availability in many tropical caves is comparatively stable over time due to reduced seasonality (Hüppop 2000). Hence, cave populations of *P. mexicana* may have adapted to perpetual rather than temporal shortages of energy. In the cave habitats investigated here, continuous supply of food is mediated by bat colonies depositing guano (in both caves) and by chemoautotrophic primary production by sulfide-oxidizing bacteria (in the sulfidic cave; Roach et al. 2011). Interestingly, fish from the nonsulfidic cave lacking any sort of primary production exhibited a more pronounced reduction of simulated total energy expenditure than fish from the sulfidic cave; hence, overall energy availability is likely a key determinant of metabolic rate evolution in this system.

Cave fish having higher routine metabolism than their surface counterparts also poses the question of whether increased energy consumption was caused by behavioral differences. Poeciliids generally are diurnal (Coleman 2011). The darkness of the respirometry chambers could have reduced activity levels of surface fish, while cave fish remained active and maintained elevated routine metabolic rates. Indeed, cave populations of *P. mexicana* are characterized by sensory and behavioral adaptations to permanent darkness, which are absent in surface ancestors (Parzefall 2001; Plath et al. 2004; Rüschenbaum and Schlupp 2013). However, quantifying activity of fish in complete darkness indicated that cave fish did not have consistently higher activity than surface fish. Instead, fish from extreme habitats generally had a higher activity than those from the ancestral nonsulfidic surface population, and sex differences were idiosyncratic across all populations investigated (significant three-way interaction term including presence of light, presence of H_2S, and sex; see the appendix for details). Nonetheless, future studies should more rigorously test how individual variation in behavior affects met-

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abolic rates and vice versa (see Biro and Stamps 2010; Careau and Garland 2012).

Metabolic Rate Variation in Sulfidic Environments

Similar to fish in caves, fish in sulfidic habitats exhibited a body size–driven reduction in simulated total energy expenditure (appendix; fig. A2). In contrast, we did not find differences in routine metabolic rates between sulfidic and nonsulfidic populations (irrespective of whether they were located in cave or surface habitats). This contradicted previous hypotheses that predicted either lower metabolic rates (in response to energy shortage or the rampant hypoxia in sulfidic environments) or higher metabolic rate in sulfidic fish (in response to increased metabolic costs of sulfide detoxification; Riesch et al. 2011*b*). It is important to note, however, that it remains unclear how routine metabolic rates measured in our experimental setup compare with routine metabolic rates in situ because all oxygen consumption measurements were conducted in the absence of H_2S. In general, the presence of physiochemical stressors and toxicants can increase metabolic rates because coping strategies and detoxification pathways are energetically costly (Penttinen and Kukkonen 1998; Rose et al. 2006; McKenzie et al. 2007). In metazoans, H_2S detoxification is primarily linked to the sulfide:quinone oxidoreductase pathway (Griesbeck et al. 2000; Shahak and Hauska 2008), which oxidizes sulfide to less-toxic forms of sulfur while consuming energy (Ip et al. 2004; Hildebrandt and Grieshaber 2008). *Poecilia* from sulfidic habitats have consistently upregulated genes associated with H_2S detoxification both in natural habitats (J. L. Kelley, C. N. Passow, L. Arias-Rodriguez, D. P. Martin, M.-C. Yee, C. D. Bustamante, and M. Tobler, unpublished data) and on experimental sulfide exposure in the laboratory (Tobler et al. 2014). However, it remains to be tested whether exposure to H_2S increases metabolic rates in *P. mexicana* in a similar fashion as in some sulfide-adapted invertebrates (Gorodezky and Childress 1994; Schneider 1996), in which case the present study would have overestimated differences in overall energy consumption between sulfidic and nonsulfidic populations. Because H_2S also blocks cytochrome c oxidase (COX) in the mitochondrial respiratory chain (Cooper and Brown 2008), sulfide exposure can also cause metabolic rate depression (Brauner et al. 1995; Blackstone et al. 2005; Volpato et al. 2008), and this may be particularly relevant for the populations investigated here. Unlike other evolutionarily lineages of sulfide spring *Poecilia* in southern Mexico that have evolved H_2S-resistant COXs, sulfide spring populations in the Tacotalpa drainage used in the present study exhibit an H_2S-susceptible COX similar to that found in ancestral nonsulfidic populations (Pfenninger et al. 2014). Consequently, there is also a possibility that our study actually underestimated differences in energy consumption between sulfidic and nonsulfidic populations in their natural habitats, and future experiments will need to isolate the potential effects of H_2S exposure in driving metabolic rate variation in natural populations.

Conclusions

Variation in metabolic rates is central to several physiological and ecological theories (e.g., Kooijman 2000; Brown et al. 2004), but we know comparatively little about the microevolutionary mechanisms that drive macroevolutionary patterns of metabolic rate variation. Our study indicates that adaptation to extreme environmental conditions is manifested in changes in energy metabolism (Parsons 1996), leading to striking intraspecific variation in energetic demands at small spatial scales. Notably, extremophiles have consistent reductions in body size in natural habitats that drive an overall reduction of energy demands. Hence, environmentally induced changes in energy supply and demand may be a major diving force in metabolic rate evolution (Mueller and Diamond 2001).

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