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Reports

Hydrogen sulfide, bacteria, and fish: a unique, subterranean food chain

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Abstract. Photoautotrophs are generally considered to be the base of food webs, and habitats that lack light, such as caves, frequently rely on surface-derived carbon. Here we show, based on analysis of gut contents and stable isotope ratios of tissues (^{13}C:^{12}C and ^{15}N:^{14}N), that sulfur-oxidizing bacteria are directly consumed and assimilated by the fish *Poecilia mexicana* in a sulfide-rich cave stream in Tabasco state, Mexico. Our results provide evidence of a vertebrate deriving most of its organic carbon and nitrogen from in situ chemoautotrophic production, and reveals the importance of alternative energy production sources supporting animals in extreme environments.

Key words: cave fish; chemoautotroph; food web; hydrogen sulfide; Poecilia mexicana; *production source*.

Introduction

The organic matter and energy that moves through food webs has been assumed to originate solely from plants and decomposers that transform organic matter via the microbial loop (Naeem 2002). In the absence of light to support photosynthesis, subterranean life is frequently assumed to be supported by consumption of surface-derived carbon (Griebler 2001, Alfreider et al. 2003). Previous studies using stable isotope analysis, however, have demonstrated that subterranean macroinvertebrates can obtain organic carbon from chemoautotrophic producers that oxidize methane in aquifers (Opsahl and Chanton 2006), lakes (Bunn and Boon 1993, Deines et al. 2009), and streams (Kohzu et al. 2004). Similarly, carbon fixed through the oxidation of hydrogen sulfide (H_2S) provides the foundation for food chains supporting macroinvertebrates and vertebrates in caves (Sarbu et al. 1996) and deep-sea hydrothermal vents and seeps (Van Dover 2002, MacAvoy et al. 2008). Notably, studies have yet to document any vertebrates that directly consume microbial chemoautotrophs. Here we provide evidence of direct consumption and assimilation of chemoautrophic bacteria by a cave-dwelling fish.

The Cueva del Azufre (Sulfur Cave) system in Mexico consists of a unique set of stream habitats with all combinations of exposure to light and toxic H_2S: a sulfidic cave stream within the Cueva del Azufre, the sulfidic surface stream El Azufre (which flows out of the sulfur cave and is fed by additional sulfide springs at the surface), a non-sulfidic cave stream within the Cueva Luna Azufre, and various non-sulfidic surface streams and rivers (see Plate 1). All stream types have been colonized by the detritivorous, live-bearing fish *Poecilia mexicana* (Poeciliidae). Despite the lack of physical barriers, each habitat type harbors a distinct morphotype of *P. mexicana*, and gene flow among populations residing in different habitats is low, indicating that fish do not migrate among adjacent habitat types (Tobler et al. 2008). However, the caves are connected to the surface by openings large enough to permit some degree of passive or active transport of organic material into the subterranean systems. Terrestrial material can fall into caves through skylights, and guano from bat colonies is abundant in both sulfidic and non-sulfidic caves (Tobler 2008). Photosynthetic primary production is absent in caves (Poulson and Lavoie 2000) and may be reduced in sulfidic streams because of H_2S toxicity (Bagarinao 1992). The sulfur-rich habitats support dense, white mats of chemoautotrophic bacteria, including *Thiobacilli* spp. and *Acidimicrobium ferrooxidans* (Hose et al. 2000). These bacteria biosynthesize energy-rich organic molecules using energy derived from the oxidization of H_2S with sulfuric acid (H_2SO_4) as a byproduct (Hose et al. 2000). In addition, green and purple sulfate-reducing bacteria, such as *Desulfobulbus propionicus*, are present, and these taxa frequently produce elemental sulfur as an end product (Hose et al. 2000).

We analyzed gut contents and stable isotope signatures of primary producer and metazoan tissues (ratios of ^{13}C:^{12}C and ^{15}N:^{14}N) to estimate the production sources assimilated by *P. mexicana* in the different habitat types. We were mainly interested in determining if chemoautotrophic bacteria contribute to *P. mexicana* biomass in the sulfur-rich surface and cave streams.

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Study Area

The study was conducted in August of 2008 in southern Tabasco state, Mexico, near the village of Tapijulapa (see Plate 1 for exact locations). The habitats sampled include the non-sulfidic surface streams Arroyo Tacubaya and Río Oxolotan, two sites in the sulfidic surface stream El Azufre, one site in the non-sulfidic cave stream Cueva Luna Azufre, and two sites in the sulfidic cave stream Cueva del Azufre. The non-sulfidic surface stream (Arroyo Tacubaya) drains into the Río Amatan, and the sulfidic surface stream drains into the Río Oxolotan (the two rivers meet to form the Río Tacotalpa approximately 2 km downstream of the sampling sites). All surface streams are at least partially shaded. Both of the cave streams are fed by springs and are drained by the sulfidic surface stream. Guano from the Ghost-faced bat, *Mormoops megalophylla*, is available to fishes in both the caves. The sulfidic surface and cave streams are characterized by dissolved H_2S concentrations of up to 300 μmol/L and low concentrations of dissolved oxygen (Hose et al. 2000, Tobler et al. 2006, Plath et al. 2010). The non-sulfidic surface and cave streams have H_2S concentrations below the detection threshold and dissolved oxygen concentrations of up to 4.3 mg/L (Tobler et al. 2006, Plath et al. 2010). In the surface stream habitats, samples were collected along approximately 50 m of stream, which included both riffle and pool habitats. In the non-sulfidic cave stream, all samples came from a single pool of water containing fish in the main cave chamber. Finally, in the sulfidic cave stream, samples were collected in cave chambers V and X (see Parzefall 2001), where both riffle and pool habitat is available.

Methods

Sample collections

Gut contents of *P. mexicana* were analyzed from all sites, and stable isotope ratios of metazoan tissues and potential basal food web elements were analyzed for one site per habitat type. Samples of leaves from the dominant vegetation (mosses, ferns, *Acacia*, *Heliconia*, and grasses; $n = 10$) were collected from the riparian zone of the surface streams. Small seedlings ($n = 2$), which had apparently been carried in by bats and germinated in the dark, were collected from the non-sulfidic cave. Snails ($n = 17$) were collected by hand from each habitat as a proxy for the stable isotope signature of benthic algae or other predominant biofilms (Vander Zanden and Rasmussen 1999). Mats of sulfur-oxidizing bacteria ($n = 4$) were collected from the sulfidic habitats. Samples of bat feces ($n = 6$), a potentially important source of carbon for cave fishes (Tobler 2008), were obtained from the caves. Fishes were collected from all habitats with a seine, anesthetized with tricaine methanesulfonate, and preserved in 10% formalin for gut contents analysis after removal of a sample of muscle tissue ($n = 20$) from the dorso-lateral region with a scalpel. The aquatic hemipteran *Belostoma* sp. ($n = 9$) was collected from the non-sulfidic cave, and samples of dipteran larvae were taken from the non-sulfidic cave stream ($n = 4$) and the sulfidic surface stream ($n = 5$). Samples of snails and dipteran larvae were composites of several individuals to ensure adequate material for mass spectrometry. All samples were placed in plastic bags with salt, which has little influence on stable isotope signatures of tissues (Arrington and Winemiller 2002), for later processing at Texas A&M University.

Gut contents analysis

Gut contents analysis was used to elucidate food resources ingested by *P. mexicana* from the different habitat types. Formalin-preserved specimens were dissected, and proportions of dietary items in the foregut were quantified (methods in Winemiller [1990]). We recognized the following food categories: detritus, algae (predominantly filamentous algae and diatoms), sulfur bacteria, invertebrates (predominantly the dipteran larvae *Goeldichironomus fulvipilus* and small snails), and bat guano (shredded insect parts). Because *P. mexicana* is not capable of masticating and reducing insects into smaller parts, we were able to distinguish insect fragments from the consumption of bat guano from insects that had been consumed whole. Accordingly, insect fragments (chitin) were classified as bat guano. Chemoautotrophic sulfide-oxidizing bacteria were clearly identifiable in gut contents in the form of dense aggregations of white filaments. Most fish also had sand in their guts, but because sand cannot be assimilated, it was excluded from statistical analyses.

For data analysis, volumetric proportions of each dietary category were arcsine-square-root transformed and then subjected to a multivariate analysis of covariance with habitat type and site (nested within habitat type) as factors. F ratios were approximated using Wilks' lambda. Assumptions of normal distribution and homogeneities of variances and covariances were met for this analysis. For visualization of the gut contents data, we performed a correspondence analysis in CANOCO (version 4.5; ter Braak and Smilauer 2002). Correspondence analysis scores from the first two axes were averaged for each site and plotted in Fig. 1.

Stable isotope analysis

Samples of primary producers, bacteria mats, fishes, and insects were rinsed and then soaked in deionized water for four hours to remove salt. The shells were removed from snail samples by hand. All samples were subsequently dried at 65°C for 48 hours and ground to a fine powder using a mortar and pestle. Subsamples were then weighed into Ultra-Pure tin capsules (Costech Analytical, Valencia, California, USA) and sent to the W. M. Keck Paleoenvironmental and Environmental Stable Isotope Laboratory (University of Kansas, Lawrence, Kansas, USA) for analysis of carbon and nitrogen isotope ratios using a ThermoFinnigan MAT

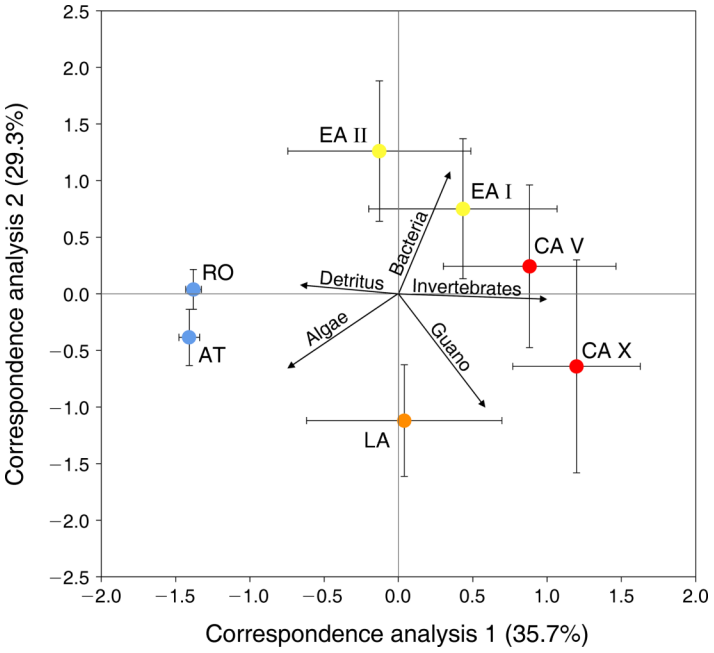

FIG. 1. Scores (mean ± SD) of a correspondence analysis on dietary items in gut contents of *Poecilia mexicana* from different sites. Non-sulfidic surface habitats (AT, Arroyo Tacubaya; RO, Rio Oxolotan) are indicated in blue, sulfidic surface stream (EA I, El Azufre, cave resurgence; EA II, El Azufre, big spring) in yellow, the sulfidic cave stream (CA, Cueva del Azufre, cave chambers V and X) in red, and the non-sulfidic cave stream (LA, Cueva Luna Azufre) in orange.

253 continuous-flow mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The standard was Pee Dee Belemnite limestone for carbon isotopes and atmospheric nitrogen for nitrogen isotopes.

The MixSIR model (Moore and Semmens 2008) was used to estimate the relative contribution of production sources assimilated by *P. mexicana*. This model uses a Bayesian framework to calculate proportional contributions of production sources from 0% to 100% while accounting for uncertainty associated with multiple sources, fractionation, and isotope signatures (Moore and Semmens 2008). Models were run separately for each habitat using in situ samples of production sources, *P. mexicana*, and aquatic invertebrates. Samples were not corrected for lipids because C:N ratios were relatively low (mean *P. mexicana* C:N = 3.6, mean aquatic invertebrate C:N = 4.5). We accounted for trophic fractionation of $\delta^{15}N$ using values from a synthesis of field and laboratory measurements of fractionation in herbivorous fishes and invertebrates (mean and standard deviation of 2.5‰; Vander Zanden and Rasmussen 2001). The trophic level (TL) of *P. mexicana* was calculated for all of the habitats based on the equation from Adams et al. (1983):

$$\mathrm{TL} = \sum_{j=1}^{n} T_j(P_{ij}) + 1$$

where T_j is the trophic position of prey species j and P_{ij} is the volumetric proportion of consumed food of species i feeding on prey species j. This method calculates plants as TL 1.0. Since it is unclear whether *P. mexicana* can actually absorb nutrients from insects present in bat guano, which is comprised of fragments of exoskeleton, we calculated trophic position for cave models both with and without bat guano. We assumed a TL of 2.0 for dipteran larvae and TL of 3.0 for *Belostoma* sp. Differences in $\delta^{13}C$ among the streams were small for samples of C_4 grasses (non-sulfidic stream value =

TABLE 1. Relative frequencies (± SD) of volumetric proportions of different food items found in *Poecilia mexicana* from different habitats.

Site	H_2S	Light exposure	*N*	Detritus	Sulfur bacteria	Algae
Cueva del Azufre, chamber V	+	−	38	0.10 ± 0.12	0.19 ± 0.25	<0.01 ± <0.01
Cueva del Azufre, chamber X	+	−	40	0.03 ± 0.06	0.07 ± 0.12	<0.01 ± <0.01
El Azufre, cave resurgence	+	+	33	0.21 ± 0.21	0.27 ± 0.29	0.01 ± 0.03
El Azufre, big spring	+	+	41	0.37 ± 0.29	0.35 ± 0.29	0.01 ± 0.02
Cueva Luna Azufre	−	−	41	0.28 ± 0.27	<0.01 ± <0.01	0.17 ± 0.21
Arroyo Tacubaya	−	+	28	0.73 ± 0.16	<0.01 ± <0.01	0.23 ± 0.17
Rio Oxolotan	−	+	30	0.69 ± 0.14	<0.01 ± <0.01	0.02 ± 0.03

Notes: Note that sand made up the remaining proportion of gut contents. Trophic levels were calculated both with and without insects derived from bat guano. The symbols + and − indicate presence or absence of hydrogen sulfide and light in each of the habitats. *N* is the number of samples.

TABLE 2. Median and 5–95% confidence percentiles (in parentheses) of estimated source contributions to *P. mexicana* in each stream habitat.

Habitat	Bacteria	Benthic algae	Bat guano	C_3 plant	C_4 grass
A. Cueva del Azufre, chamber V	0.52 (0.41–0.59)	NP	0.04 (<0.01–0.59)	0.40 (0.33–0.47)	0.03 (<0.01–0.10)
B. El Azufre, big spring	0.21 (0.02–0.61)	NP	NP	0.70 (0.36–0.86)	0.09 (0.01–0.19)
C. Cueva Luna Azufre	NP	NP	<0.01 (0–0.02)	0.78 (0.76–0.80)	0.21 (0.20–0.23)
D. Arroyo Tacubaya	NP	0.07 (<0.01–0.20)	NP	0.90 (0.78–0.96)	0.03 (<0.01–0.06)

Notes: Stream types sampled include: A, sulfidic cave; B, sulfidic surface; C, non-sulfidic cave; and D, non-sulfidic surface. NP indicates that the production source was not present in that habitat.

−13.4‰, sulfidic stream value = −13.3‰), therefore models were run using the average value for this production source. However, among-habitat variation in $\delta^{13}C$ was high for C_3 macrophytes (ANOVA, $F_{2,7} = 8.20$, $P < 0.05$), thus C_3 macrophyte values were not combined. Because the sulfidic surface stream is adjacent to both caves, which lack plant life, the sulfidic surface stream C_3 macrophyte mean was used for the sulfidic cave stream model. Among-habitat variation for snails was high for both $\delta^{13}C$ (ANOVA, $F_{3,12} = 11.26$, $P < 0.001$) and $\delta^{15}N$ (ANOVA, $F_{3,12} = 168.71$, $P < 0.0001$). We used the mean $\delta^{13}C$ and $\delta^{15}N$ signature of snails from the non-sulfidic stream site as the stable isotope signature of benthic algae after accounting for trophic fractionation of $\delta^{15}N$ (Vander Zanden and Rasmussem 2001). Samples of bacterial mats collected from the sulfidic stream were more enriched in ^{13}C (−1.0–0.2‰) compared to the sample collected from the sulfidic cave (−7.1‰), so these values were not combined for the sulfur-rich habitat models. Bat guano was more depleted in ^{13}C and ^{15}N in the non-sulfidic cave (mean $\delta^{13}C = -28.4$, mean $\delta^{15}N = 6.5$) compared to the sulfidic cave (mean $\delta^{13}C = -24.5$, mean $\delta^{15}N = 7.9$) and therefore were not combined for the cave models. We resampled sulfidic cave stream, sulfidic surface stream, and non-sulfidic surface stream models a total of 100 000 times. Non-sulfidic cave stream models were resampled a total of 1 000 000 times. For all of the models, the maximum importance ratio was <0.0001, and there were >1000 posterior draws, indicating that the true posterior density was effectively estimated. Cave models using TL calculated with insect fragments were essentially the same as models that eliminated insect fragments from TL calculations; therefore we only present cave models based on TL calculated with insects derived from bat guano.

TABLE 1. Extended.

	Trophic level	
Invertebrates	With insects	Without insects
0.38 ± 0.36	2.4 ± 0.4	2.3 ± 0.3
0.44 ± 0.41	2.4 ± 0.4	2.2 ± 0.3
0.27 ± 0.28	2.3 ± 0.3	2.3 ± 0.3
0.08 ± 0.19	2.1 ± 0.2	2.1 ± 0.2
0.47 ± 0.30	2.5 ± 0.3	2.2 ± 0.3
<0.01 ± <0.01	2.0 ± 0.0	2.0 ± 0.0
<0.01 ± <0.01	2.0 ± 0.0	2.0 ± 0.0

RESULTS

Poecilia mexicana gut contents were variable among habitat types (Table 1). Multivariate analysis of variance (MANOVA) of dietary items indicated significant differences among habitats (Fig. 1; $F_{12,638} = 46.25$, $P < 0.001$) as well as site-specific variation within habitats (site nested within habitat: $F_{12,638} = 9.96$, $P < 0.001$). Whereas fish in non-sulfidic surface habitats primarily ingested detritus and algae, conspecifics in the sulfidic surface and cave streams had diets dominated by chemoautotrophic bacteria and aquatic invertebrates, suggesting that the organic matter assimilated by fish in sulfidic habitats could derive from chemoautotrophic primary producers. Fish TL calculated with insects derived from bat guano ranged from 2.0 to 2.5, and TL calculated without insect fragments ranged from 2.0 to 2.3 (Table 1).

Among-habitat variation in stable isotope ratios of fish was high for both $\delta^{13}C$ (ANOVA, $F_{3,16} = 43.49$, $P < 0.001$, Fig. 2) and $\delta^{15}N$ (ANOVA, $F_{3,16} = 179.98$, $P < 0.001$). Tukey's multiple comparisons test indicated that $\delta^{13}C$ of *P. mexicana* was significantly different among all streams except for those in sulfidic and non-sulfidic caves. Fish from the sulfidic and non-sulfidic cave streams were most enriched in ^{13}C (higher $\delta^{13}C$ values), and fish from the non-sulfidic cave stream were most depleted. Additionally, fish from the sulfidic cave stream were significantly more depleted in ^{15}N (lower $\delta^{15}N$ values) compared to fish from all other habitats (Fig. 2).

The sulfidic cave stream MixSIR model estimated that *P. mexicana* assimilated carbon and nitrogen primarily from chemoautotrophic bacteria (median contribution = 53%, 5% and 95% confidence percentiles = 0.41% and 0.59%; Table 2, Appendix) followed by C_3 plants (median contribution = 40%, 5% and 95% confidence percentiles = 0.33% and 47%). In contrast, *P. mexicana* in all other habitat types assimilated material mostly from C_3 plants; median contributions ranged from 0.70% in the sulfidic surface stream to 0.90% in the non-sulfidic surface stream. The MixSIR model indicated that chemoautotrophic bacteria made a small contribution to fish in the sulfidic surface stream as well (median contribution = 0.21%, 5% and 95% confidence

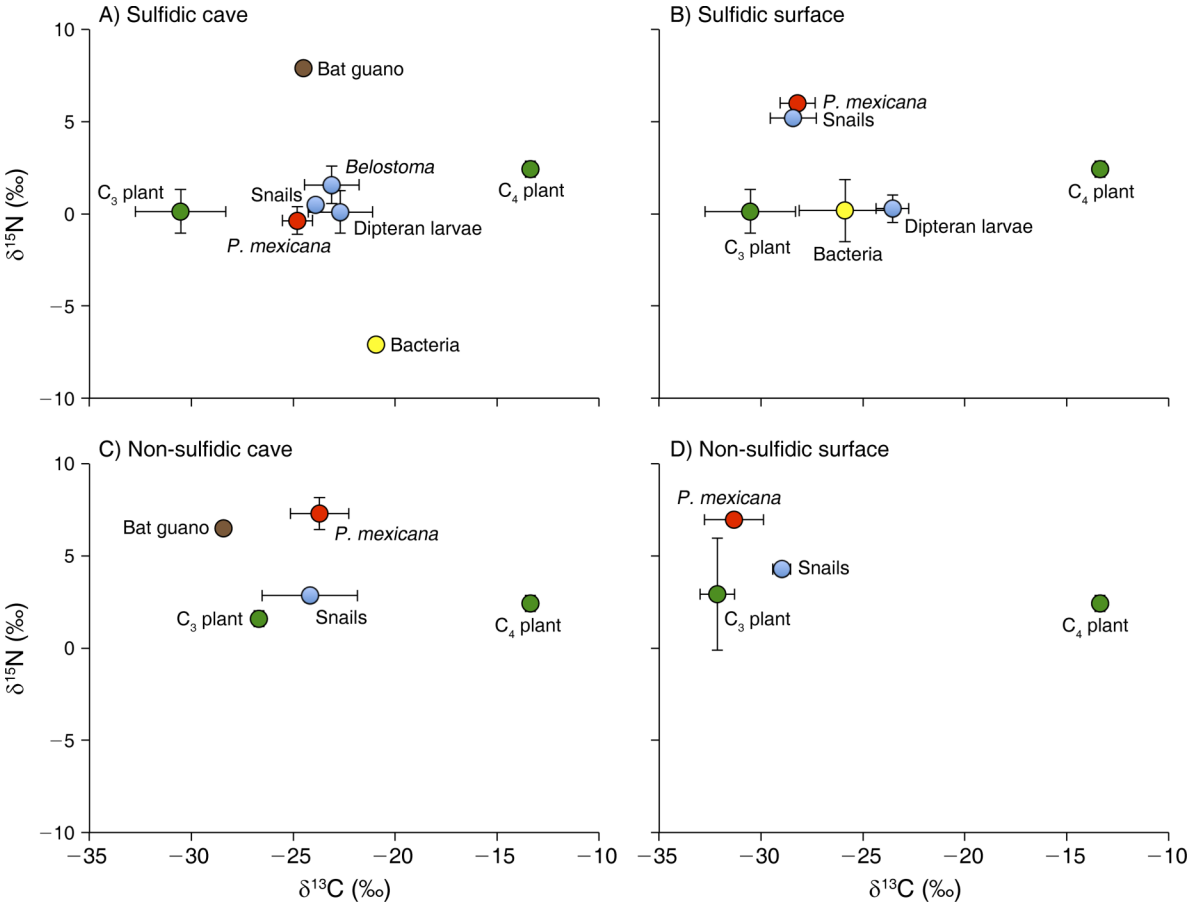

FIG. 2. Stable isotope ratios (mean ± SD) for *P. mexicana* and production sources in the (A) sulfidic cave, (B) sulfidic surface habitat, (C) non-sulfidic cave, and (D) non-sulfidic surface habitat. Plant primary producers are highlighted in green, bacteria in yellow, bat guano in brown, fish in red, and other consumers in blue.

percentiles = 0.02% and 0.61%), but the diagnostic histogram was slightly right skewed, indicating that the model may have been inefficient in approximating posterior distributions. In the sulfidic cave stream, chemoautotrophic bacteria also were an important basal production source supporting the predatory hemipteran *Belostoma* sp. (median contribution = 0.47%, 5% and 95% confidence percentiles = 0.38% and 0.56%) and dipteran larvae (median contribution = 0.36%, 5% and 95% confidence percentiles = 0.23% and 0.49%).

DISCUSSION

Food webs in unshaded, autotrophic streams are frequently based on algal production sources because they have more nutritional value and are less recalcitrant than tissues of most macrophytes (Rounick et al. 1982, McCutchan and Lewis 2002). However, in heavily shaded, heterotrophic streams, the availability of algae decreases and terrestrial-based production sources, such as dissolved organic carbon (DOC; Meyer et al. 1997, Hall and Meyer 1998) and leaf litter (Wallace et al. 1999, Hall et al. 2000), are more important. Streams in caves are almost always heterotrophic because of the absence of light. Consequently, many metazoans are dependent on terrestrial-based production sources, such as biofilms fueled by DOC (Culver 1985, Simon et al. 2003) and bat guano (Harris 1970, Ferreira and Martins 1999). In caves with sufficient inputs of solute-rich groundwater, chemoautotrophic production can provide an additional source of organic carbon (Sarbu et al. 1996, Opsahl and Chanton 2006).

Our results indicated that fish in the non-sulfidic streams, both above- and belowground, appeared to be supported almost entirely by photosynthetic primary production. In the non-sulfidic cave, carbon is imported through bat guano deposition and detritus in runoff from surface habitats. In the sulfidic surface stream, plants primarily support metazoan biomass despite the presence of sulfur bacteria. In contrast, in the sulfidic cave stream, fish and aquatic invertebrates obtained comparatively little material from detritus derived from photoautotrophs. Both gut contents and stable isotope analyses indicated that most of the carbon and nitrogen obtained by fish from the sulfidic Cueva del Azufre stream derives from in situ chemoautotrophic production.

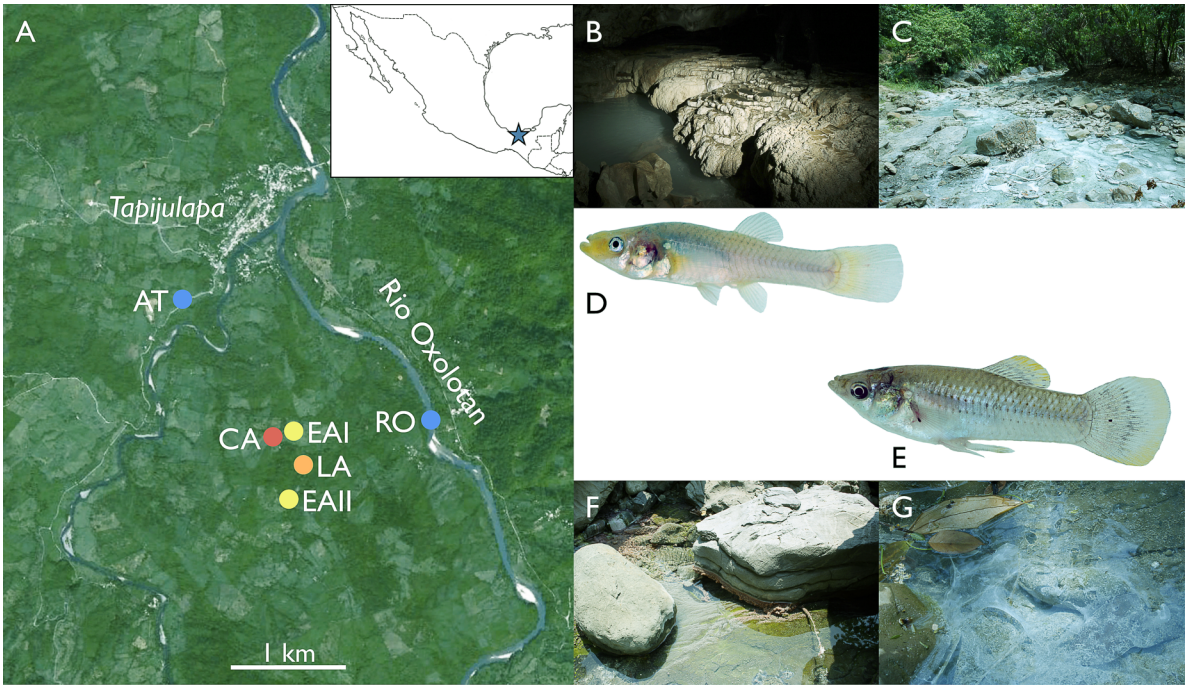

PLATE 1. (A) Map of the study site near the village of Tapijualapa in southern Tabasco state, Mexico. Non-sulfidic surface habitats Arroyo Tacubaya (AT) and Rio Oxolotan (RO) are indicated in blue; sulfidic surface stream Azufre (EAI), cave resurgence El Azufre, and big spring (EAII) in yellow; the entrance to the sulfidic cave stream Cueva del Azufre (CA) in red; and the entrance to the non-sulfidic cave stream Cueva Luna Azufre (LA) in orange. Latitude (°N)/longitude (°W) of habitats: AT, 17.4536/92.7845; RO, 17.4444/92.7629; EAI, 17.4423/92.7745; EAII, 17.4384/92.7748; CA cave entrance, 17.17.4423/92.7754; LA cave entrance, 17.4417/92.7731. (B) View into the Cueva del Azufre. (C) View of the sulfidic surface stream. (D) Female cavefish from the Cueva del Azufre with reduced eye size and pigmentation. (E) Male fish from the sulfidic surface stream. (F) Colonies of purple and (G) white sulfide bacteria growing in the sulfidic surface stream. Photo credits: M. Tobler.

The assimilation of chemoautotrophic bacteria by fish in the sulfidic cave stream yielded significantly lower $\delta^{15}N$ values compared to fish from all the other habitats. The differences in $\delta^{15}N$ values of bacteria and thus fish between the sulfidic cave stream and the sulfidic surface stream could have been due to variation in the nitrogen cycle between the two habitats. When ammonium concentrations are high, assimilation by bacteria generally favors ^{14}N over ^{15}N, resulting in high isotope fractionation and lower values of $\delta^{15}N$ (Hoch et al. 1992, Lee and Childress 1994). Higher concentrations of ammonium in the sulfidic cave compared to the sulfidic surface stream could have contributed to lower $\delta^{15}N$ values of bacteria and thus fish. Nitrification also can deplete ^{15}N (Yoshida 1988).

Analysis of gut contents revealed that fish in the sulfidic cave stream primarily consumed sulfur-oxidizing bacteria and insects, including aquatic dipteran larvae and exoskeleton fragments from bat guano. However, differences among mean $\delta^{15}N$ of sulfur-oxidizing bacteria (−7.1), dipteran larvae (0.10), and fish (−0.4) indicate that fish had not assimilated much, if any, insect biomass. This discrepancy between gut contents and stable isotope data can be explained by the low digestibility of exoskeleton fragments observed in fish guts. Because the insects had already passed through the intestines of the bats, the remaining insect fragments from bat guano were mostly exoskeleton comprised of chitin, a highly recalcitrant polysaccharide with very low nutritional value. Our study provides evidence of a unique food chain in a sulfidic cave stream consisting of $H_2S \rightarrow$ bacteria $\rightarrow$ fish, and contributes further evidence of alternative energy production sources supporting animals in extreme environments.

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APPENDIX

Estimations of source contributions from MixSIR model for *Poecilia mexicana* from the sulfidic cave, sulfidic surface stream, non-sulfidic cave, and non-sulfidic surface stream (*Ecological Archives* E092-179-A1).