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POPULATION STRUCTURE, HABITAT USE, AND DIET OF GIANT WATERBUGS IN A SULFIDIC CAVE

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ABSTRACT—In the southern Mexican Cueva del Azufre, a cave ecosystem with high concentrations of toxic hydrogen sulfide, one species of hemipteran (*Belostoma* cf. *bakeri*) has adopted a unique ecological function in that it acts as one of the top predators in the subterranean food web, preying on the abundant cavefish *Poecilia mexicana*. To date, several aspects of this predator-prey interaction have been explored, but basic questions related to the ecology of the waterbugs remained unstudied. We tested whether there is evidence for reproduction of waterbugs within the cave, which would indicate a self-sustaining population. Furthermore, we investigated the habitat affinities of different size-classes of waterbugs. We infer that waterbugs reproduce inside the cave despite the toxic properties of the water, as evidenced by the presence of nymphs of all sizes and males carrying developing zygotes. We also found size-dependent differences in use of habitat, and particularly small nymphs occupy different microhabitats than larger individuals. Adult waterbugs and large nymphs were most common at sites with cavefish. Small nymphs were rare at these locations, possibly to avoid cannibalism or exploit different resources in other locations. Furthermore, stable-isotope-analysis indicated that waterbugs likely derive their carbon from a combination of fish, dipteran larvae, and chemoautotropic bacteria.

RESUMEN—En el sur de México en la Cueva del Azufre, un ecosistema cavernícola con altas concentraciones del tóxico sulfuro de hidrógeno, una especie hemíptera (*Belostoma* cf. *bakeri*) ha adoptado una función ecológica única, actúa como uno de los depredadores más altos de la red alimentaria subterránea, comiendo el abundante pez de la cueva, *Poecilia mexicana*. Hasta la fecha, varios aspectos de esta interacción entre el depredador y su presa han sido estudiados, pero preguntas básicas relacionadas a la ecología de *B. bakeri* se mantienen sin estudio. Examinamos si se encuentra evidencia de reproducción en *B. bakeri* adentro de la cueva, lo que indicaría que la población es autosostenible. También investigamos las afinidades del hábitat de diferentes clases de tamaño de *B. bakeri*. Dedujimos que *B. bakeri* se reproduce adentro de la cueva a pesar de las propiedades tóxicas del agua, en base a la presencia de ninfas de todos tamaños y de los machos cargando cigotos en desarrollo. También encontramos diferencias en uso de hábitat según el tamaño corporal, especialmente que las ninfas pequeñas ocupan microhábitats diferentes que individuos más grandes. Adultos y ninfas grandes fueron más comunes en sitios con peces de la cueva. Las ninfas pequeñas casi no se encontraron en estos sitios, posiblemente para evitar el canibalismo o para explotar otro tipo de recursos en otros lugares. Además, el análisis de isótopos estables indicó que *B. bakeri* probablemente deriva su fuente de carbón al ingerir peces, larva de dípteros, y bacterias quimioautotróficas.

Giant waterbugs of the family Belostomatidae are large aquatic hemipterans with extra-oral digestion that prey on a variety of invertebrates and vertebrates (Menke, 1979). They are sit-and-wait predators that catch bypassing prey with their strongly incrassate, raptorial forelegs. Upon capture, belostomatids inject toxins causing paralysis of prey and digestive enzymes causing necrosis of tissue (Swart and Felgenhauer, 2003; Swart et al., 2006). While the large-bodied species of the subfamily Lethocerinae have been widely reported to feed on large prey including small snakes, anurans, and most notably fish (Babbitt and

Jordan, 1996; Mori and Ohba, 2004; Ohba and Nakasuji, 2006; Swart et al., 2006), the smaller-bodied species of the subfamily Belostomatinae prey on various terrestrial and aquatic invertebrates and anurans but usually avoid fish (Cullen, 1969; Swart and Felgenhauer, 2003; Ohba and Nakasuji, 2006; Swart et al., 2006).

Among the most unusual environments inhabited by giant waterbugs is a Mexican sulfur cave, the Cueva del Azufre (Gordon and Rosen, 1962). The stream within this cave is fed by a number of springs containing toxic hydrogen sulfide (Tobler et al., 2006). Despite the toxic
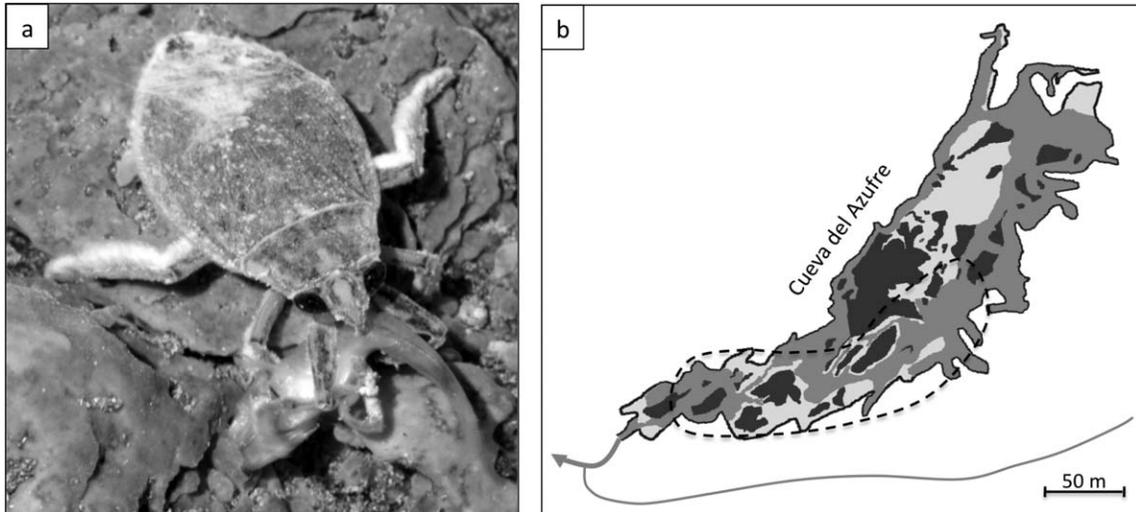
Fig. 1—a) *Belostoma* are predators of cavefish. Depicted is a waterbug (28 mm) foraging on a cavefish in situ. The white coating on the back of the abdomen and legs of the waterbug are filaments of sulfide bacteria. b) Sketch of the study area (surveyed area outlined by dashed line) depicting the major watercourses in the Cueva del Azufre. Light gray areas are dry land within the cave, dark areas indicate impenetrable bedrock, and areas in intermediate gray shading represent aquatic habitats.

conditions, the cave harbors a diverse fauna of terrestrial and aquatic organisms (Summers Engel, 2007), including a unique population of cavefish (cave mollies, *Poecilia mexicana*, Poeciliidae). These fish, similar to many other cave organisms (Porter and Crandall, 2003), exhibit reduced eye-size and pigmentation of the body but also have evolved enhanced nonvisual senses and are able to communicate in darkness (Plath et al., 2004; Tobler et al., 2008*b*, 2008*c*). Within the Cueva del Azufre, giant water-bugs of the genus *Belostoma* (subfamily Belostomatidae) and cave mollies exhibit a unique predator-prey relationship (Fig. 1a). Due to the toxic effects of hydrogen sulfide and the extreme hypoxia in the water, cave mollies spend long periods of time performing aquatic surface respiration (skimming the micro-layer of water at the surface with relatively higher concentration of dissolved oxygen and passing it over the gill filaments during opercular ventilation). While performing aquatic surface respiration, cave mollies are vulnerable to attacks by giant waterbugs lurking at the surface of the water (Tobler et al., 2009). Waterbugs preferentially prey upon large-bodied individuals over small ones (Plath et al., 2003; Tobler et al., 2007), gestating over nongestating females (Plath et al., 2011), and males over females (Tobler et al., 2008*a*). Consequently, waterbugs potentially affect the structure of populations of cavefish and the evolution of life histories. Furthermore, waterbugs also select against individuals that migrate between habitats inside and outside caves, thus driving divergence and reproductive isolation among populations of fish (Tobler, 2009).

While the ecology of cave mollies is well understood (see Plath and Tobler, 2010, for a review), very little is known about the population of *Belostoma* within the Cueva del Azufre. A previous study estimated the density of adult *Belostoma* in one of the front chambers of the Cueva del Azufre (Tobler et al., 2007), but it is still unclear whether the waterbugs reproduce within the cave. Alternatively, the cave with its high densities of fish might simply be a temporary feeding habitat for adults, or a population sink. We consequently conducted a thorough survey to estimate the structure of the population of *Belostoma* within the cave and searched for signs of recruitment of juvenile waterbugs inside the cave and for males carrying eggs. In the subfamily Belostomatinae, males exhibit post-copulatory paternal care as females lay their eggs directly on the back of males, and males carry the eggs until developed offspring hatch (Lauck and Menke, 1961; Kruse, 1990; Gilg and Kruse, 2003). Hence, reproduction in waterbugs is relatively easy to detect. In addition, we analyzed use of microhabitat by the water-bugs and particularly tested for ontogenetic shifts. Because cannibalism can be intense in giant waterbugs (Ohba et al., 2006; Swart et al., 2006), nymphs may avoid microhabitats preferred by larger conspecifics, or juveniles may exploit different resources underrepresented in the microhabitats with larger waterbugs. Finally, even though *Belostoma* readily feed on cavefish in an experimental setting and can occasionally be observed in situ capturing fish (Fig. 1a), it is unclear to what extent the giant waterbugs rely on fish as an energy source, and whether alternative resources are utilized. Hence, we used stable isotope ratios of carbon (C^{13}/C^{12}) and nitrogen (N^{15}/N^{14}) to determine the contribution of cavefish to the diet of *Belostoma*.

Materials and Methods—The Cueva del Azufre is located near the village of Tapijulapa in the Southern Mexican State of Tabasco. It is a relatively small cave of ca. 200-m depth from the resurgence of the cave creek to the innermost cave chamber.

However, there is a multitude of passages, and ca. 1,900 m of aquatic and terrestrial habitats have been mapped by Hose and Pisarowicz (1999). The cave is structured into different chambers, the nomenclature of which follows Gordon and Rosen (1962). The front chambers obtain some dim light through skylights, whereas the rearmost chambers are completely dark. The cave is drained by a creek fed by a number of springs, most of which contain high levels of dissolved H_2S (Tobler et al., 2006). *Belostoma* and fish occur throughout the cave, and, for this study, we focused our effort on the front section of the cave (chambers I through VI, see Fig. 1b). In theory, *Belostoma* can move into and out of the cave by flying through the skylights in the front part of the cave or by moving through the water where the stream exits the cave.

The taxonomic identity of the species of *Belostoma* in the Cueva del Azufre remains to be clarified in detail. According to keys provided by Lauck (1962, 1964), the species belongs to the *B. flumineum* species group and bears close resemblance with *B. bakeri*. However, *B. bakeri* is typically distributed along the Pacific coast from the state of Washington southward into Mexico and is particularly common in the Sonoran Desert region, reaching as far east as Texas (Lauck, 1964). It remains to be investigated whether the range of *B. bakeri* has been underestimated, potentially due to the lack of thorough surveys in southern Mexico or whether the species occurring in the Cueva del Azufre and nearby habitats at the surface represent an undescribed species resembling *B. bakeri*. We refer to the species as *B.* cf. *bakeri*.

Three people conducted the survey of populations of *Belostoma* on 3 subsequent days between 24 and 27 March 2010. To address questions about the structure of the population and use of habitat, individual *Belostoma* were collected by hand and small dip nets. All specimens were measured to the closest millimeter from the tip of the head to the tip of the abdomen using calipers. The stage of life history (nymph or adult) was determined by the presence of wings. Additionally, we recorded the presence of eggs in reproductive males. To estimate use of habitat by *Belostoma*, we recorded the maximum depth of water within 15 cm of an individual using calibrated poles (categories of depth: <5 cm; 5–10 cm; 10–20 cm; 20–30 cm; 30–40 cm; >40 cm) as well as the substrate on which an individual resided. Rocks and mud are the dominant types of substrate within the cave, but leaf litter also is present around skylights, because ceiling breakdowns allow for organic matter to be washed in from the surface. Finally, we recorded whether cavefish were present within 15 cm of the location where the individual *Belostoma* was collected.

To avoid counting the same individuals multiple times, all specimens were transferred to large plastic boxes (82 × 40 × 12 cm) equipped with moist paper towels and rocks as hiding places. Four boxes were available, and we separated bugs roughly by size to minimize cannibalism. Bugs remained in boxes until the survey was terminated. At the end of the third day, all specimens were released back into the cave.

We calculated size-distributions of *Belostoma* collected throughout the cave and compared the size of reproductive and nonreproductive adults using an independent-samples *t*-test. To test for ontogenetic shifts in use of habitat, we compared standard lengths of *Belostoma* across different depths of water and substrates using analysis of variance (ANOVA). Standard lengths of *Belostoma* collected in presence and absence of fish were compared with an independent-samples *t*-test. All data fulfilled the assumptions of normality and homoscedasticity required for parametric analyses.

For stable-isotope-analysis, we collected adult *Belostoma* ($n = 9$ individuals), cave mollies (*Poecilia mexicana*; $n = 5$ individuals), dipteran larvae ($n = 3$ pooled samples of multiple individuals of *Goeldichironomus fulvipilus*), snails ($n = 1$ pooled sample of multiple individuals of a Lymnaeidae species), and bacterial mats ($n = 1$). These samples provide a representation of the most abundant organisms within the sulfidic stream in the cave. Chemoautotrophic bacteria, including the sulfide-oxidizing bacteria *Thiobacilli* and *Acidimicrobium ferrooxidans*, are the base of the food web of this cave (Roach et al., 2011). Green and purple sulfate-reducing bacteria, such as *Desulfobulbus propionicus,* also are present in the cave (Hose et al., 2000). Samples of snails and dipteran larvae were composites of several individuals to ensure sufficient material for mass spectrometry. After collection, all samples were immediately placed in plastic bags with salt for preservation and later processing in the laboratory. Preservation with salt has little influence on stable isotope signatures of tissues (Arrington and Winemiller, 2002).

Before analysis, all samples were rinsed and then soaked in deionized water for 4 h to remove salt. The shells were removed from snails by hand. All samples were dried at 65°C for 48 h and ground to a fine powder using a mortar and pestle. Subsamples were then weighed into Ultra-Pure tin capsules (Costech Analytical, Valencia, California) and sent to the W. M. Keck Paleoenvironmental and Environmental Stable Isotope Laboratory (University of Kansas, Lawrence, Kansas) for analysis of carbon and nitrogen isotope ratios using a ThermoFinnigan MAT 253 continuous-flow mass spectrometer. 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 *Belostoma*. This model uses a Bayesian framework to calculate proportional contributions of production sources from 0–100%, while accounting for uncertainty associated with multiple sources, fractionation, and isotope signatures (Moore and Semmens, 2008). We made two models of the potential production sources supporting biomass of *Belostoma*. For the first model, we used cavefish, dipteran larvae, and snails; for the second model, we used cavefish, dipteran larvae, snails, and bacterial mats. The piercing mouthparts of *Belostoma* restrict this species to a predominantly predatory life style; therefore, it is highly unlikely that *Belostoma* are feeding directly on sulfur bacteria. However, because bacterial films make up a considerable proportion of the diet of cavefish (Roach et al., 2011), *Belostoma* may be indirectly consuming considerable amounts of sulfur bacteria while feeding on cavefish. We accounted for mean and standard deviation of fractionation of $\delta^{15}N$ and $\delta^{13}C$ using values from a synthesis of measurements (field and laboratory) of fractionation in fishes and invertebrates (Vander Zanden and Rasmussen, 2001). For average fractionation, we used the mean value for carnivores (3.23 for $\delta^{15}N$, 0.91 for $\delta^{13}C$), and we used the mean value for nonherbivores for standard deviation (0.41 for $\delta^{15}N$, 1.04 for $\delta^{13}C$). We resampled the data 10^7 times. The maximum importance ratio was < 0.001, and there were 8,090 posterior draws, indicating that the true posterior density was effectively estimated.
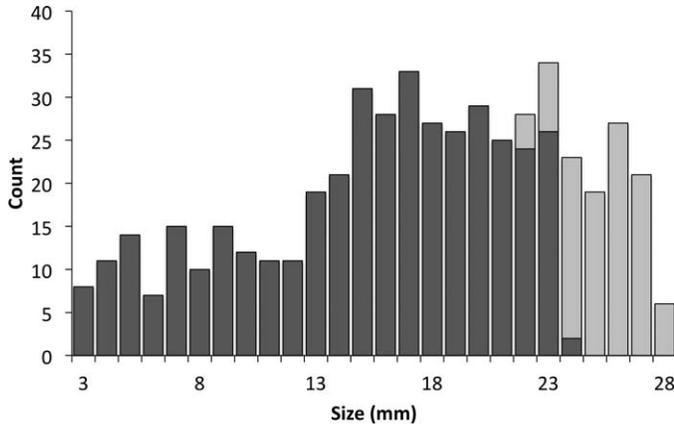
FIG. 2—Size-distribution of *Belostoma* within the front chambers of the Cueva del Azufre. Dark bars represent the number of nymphs in each size class; light bars the number of adults.

RESULTS—We collected 511 individual *Belostoma* ranging from 3–28 mm in length (mean ± SD = 17.2 ± 6.5 mm; Fig. 2). A total of 405 individuals were nymphs ranging from 3–24 mm in length (mean ± SD = 15.2 ± 5.6 mm); 106 individuals were adults ranging from 22–28 mm in length (mean ± SD = 25.4 ± 1.5 mm). Consequently, not only adults preying upon cavefish can be found in the cave, but nymphs at different developmental stages also are present. In addition, we collected 13 males carrying developing eggs, corroborating the hypothesis that water bugs reproduce inside the cave. Breeding adults were significantly larger than nonbreeding adults (independent-samples t-test, $t_{104} = -2.904$, $P = 0.005$; mean ± SD = 26.5 ± 1.0 mm and 25.2 ± 1.5 mm for breeding and nonbreeding adults, respectively).

Waterbugs were found at all depths of water from <5 to >40 cm (Fig. 3a). Small individuals were predominantly found in shallow water, whereas adults and larger nymphs were recorded at all depths. As a result, average standard length varied significantly across different categories of depth (ANOVA; $F_{5,505} = 11.060$, $P < 0.001$). In terms of substrate, the majority of *Belostoma* were collected on rocky ground, but small nymphs also were common on leaf litter (Fig. 3b). No *Belostoma* was found on muddy bottom (even though this substrate type accounts for large parts of aquatic habitat within the cave), and only a few individuals were caught swimming in the open water. As for depth of water, standard lengths varied significantly across substrates (ANOVA; $F_{2,508} = 33.274$, $P < 0.001$). Finally, occurrence of cavefish in the direct vicinity of individual waterbugs was strongly dependent on size, with small waterbugs predominantly occurring in microhabitats without fish and large ones in microhabitats with fish (Fig. 3c; independent-samples t-test: $t_{509} = -12.273$, $P < 0.001$).

Values of $\delta^{15}N$ and $\delta^{13}C$ varied within and among taxa (Fig. 4), with $\delta^{15}N$ for *Belostoma* ranging from 0.50–2.82 and $\delta^{13}C$ ranging from −24.90 to −21.57. The MixSIR
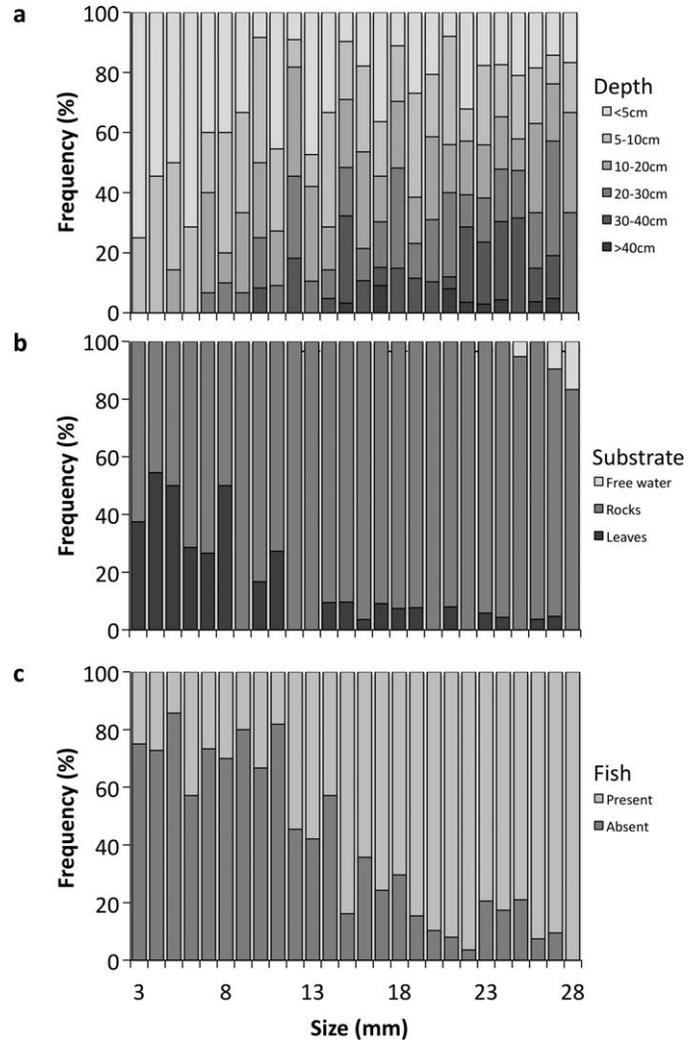

FIG. 3—Use of habitat by giant waterbugs (*Belostoma*) in the Cueva del Azufre in relation to a) depth of water, b) type of substrate, and c) presence of fish.

model with cavefish, dipteran larvae, and snails as potential production sources (Fig. 5) estimated that *Belostoma* assimilated its carbon and nitrogen primarily from dipteran larvae, with a median source-contribution of 0.858 and 5th–95th percentile contributions ranging from 0.008–0.967. Cavefish (median = 0.099, 5th–95th percentiles = 0.0008–0.973) also contributed to biomass of *Belostoma*. Due to the bimodal distribution of posterior probabilities for contributions from dipteran larvae and cavefish, however, this model could not effectively disentangle the contributions from the two sources. Snails (median = 0.025, 5th–95th percentiles = 0.002–0.103) were less important.

In contrast, the MixSIR model with cavefish, dipteran larvae, snails, and bacterial mats as potential production sources (Fig. 6) indicated that cavefish (median = 0.737, 5th–95th percentiles = 0.077–0.826) and bacteria (median = 0.176, 5th–95th percentiles = 0.121–0.228) primarily supported *Belostoma*, and that contributions of dipteran
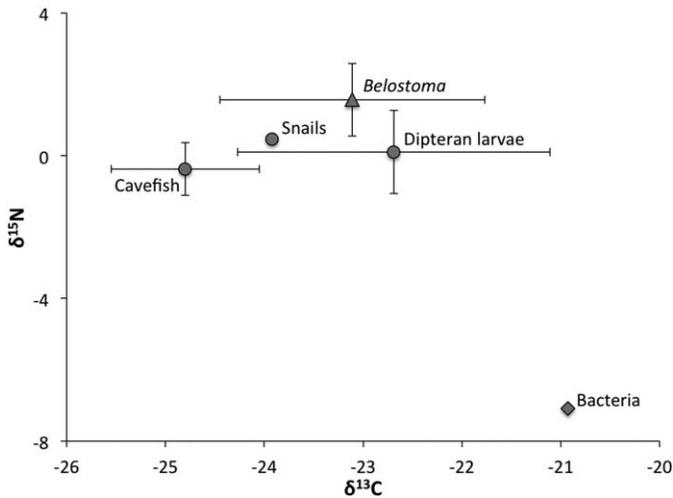
Fig. 4—Stable-isotope-ratios (mean ± *SD*) for *Belostoma* and potential sources of food in the Cueva del Azufre.

larvae (median = 0.041, 5th–95th percentiles = 0.003–0.676) and snails (median = 0.028, 5th–95th percentiles = 0.002–0.106) were much lower.

DISCUSSION—Giant waterbugs were common throughout the front chambers of the Cueva del Azufre. Using mark-recapture analysis, the size of the population of this sit-and-wait predator was estimated as 336 ± 130 (mean ± *SE*) adult individuals in cave-chamber V alone, which results in a density of >1 *Belostoma*/m^2 (Tobler et al., 2007). Our present study adds to a basic understanding of the structure of the population of *Belostoma* within the cave. Most importantly, we found evidence of reproduction and recruitment in the population in the Cueva del Azufre, because 13 males were carrying eggs. Assuming an adult sex ratio of 1:1, this corresponds to almost 25% of the adult male population guarding offspring at that time. Evidence for successful reproduction also stems from the presence of nymphs at all stages of development. The smallest *Belostoma* recorded in this study were only 3 mm in total length, which corresponds roughly to the size of offspring when hatching from the eggs (M. Tobler, pers. observ.). Consequently, the population of *Belostoma* in the Cueva del Azufre likely represents a self-sustaining population; however, it is as yet unclear whether and to what extent the population is connected to those at the surface.

Belostoma in the Cueva del Azufre exhibit clear preferences in habitat; these preferences appear to shift during the course of ontogenetic development. The great majority of large nymphs and adult waterbugs were collected while they were perching on a rocky substrate right at or close to the surface of the water and in proximity to cavefish. These waterbugs were likely lurking to prey on bypassing fish. In contrast, smaller nymphs revealed different preferences in microhabitat and were usually found on leaf litter, in shallow water, and in areas
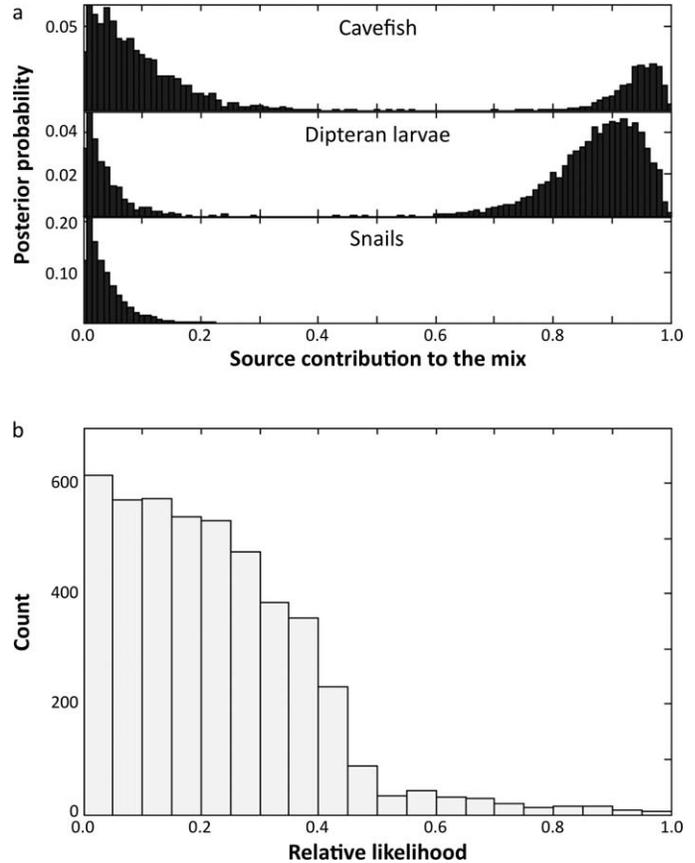

Fig. 5—a) Posterior contributions of cavefish, dipteran larvae, and snails to the biomass of *Belostoma* as estimated by the MixSIR model. b) Diagnostic histogram of the MixSIR model. The diagnostic histogram is left-skewed, indicating that posterior distributions were effectively approximated.

where fish were absent. Probably, only large terrestrial ctenid and theraphosid spiders present in the cave prey on adult *Belostoma* (Horstkotte et al., 2010); however, small nymphs are small enough to be eaten by larger cavefish and freshwater crabs (*Avotrichodactylus bidens* Bott 1969, Trichodactylidae). Furthermore, *Belostoma* have been reported to be cannibalistic (Ohba et al., 2006; Swart et al., 2006), so differential use of microhabitat by juvenile *Belostoma* could be a strategy to avoid cannibalism. Hence, the spatial distribution of juvenile *Belostoma* could be explained by cannibalism by larger conspecifics (or predation by other species) that eliminates small individuals from areas inhabited by the larger individuals. Alternatively, small *Belostoma* may have behavioral preferences for particular microhabitats that lack large conspecifics, either to avoid predation or to exploit food resources different than those consumed by large nymphs and adults.

The two stable-isotope-models included in our analysis provided somewhat conflicting results in that they differentially estimated the relative contribution of cavefish, dipteran larvae, and bacteria to the diet of *Belostoma*. Both models, however, indicated that cavefish constitute a
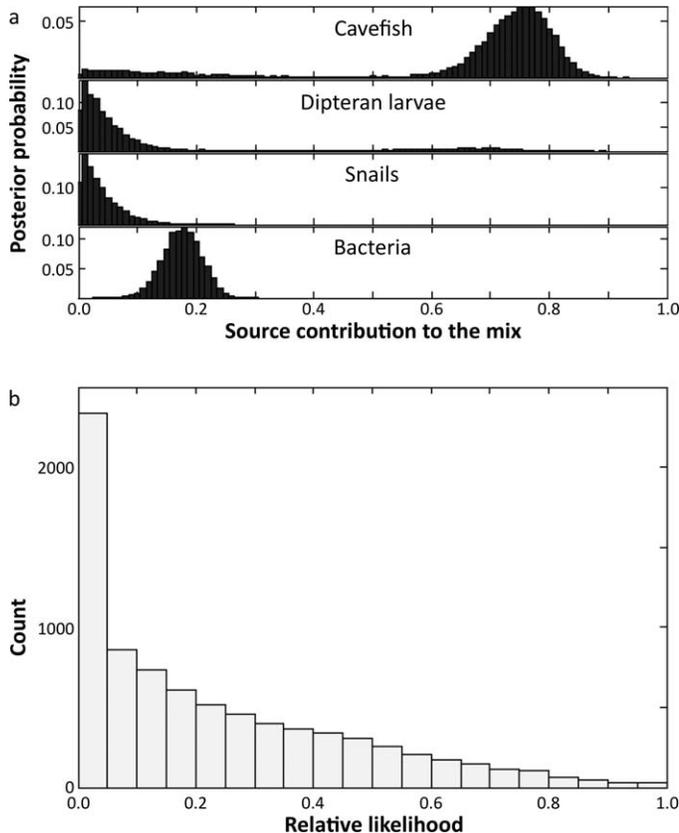
Fig. 6—a) Posterior contributions of cavefish, dipteran larvae, snails, and bacteria to the biomass of *Belostoma* as estimated by the MixSIR model, and b) diagnostic histogram of the MixSIR model. The diagnostic histogram is left-skewed, indicating that posterior distributions were effectively approximated.

substantial carbon source for *Belostoma*. Experimental and observational data also indicate that *Belostoma* prey on cavefish (e.g., Tobler et al., 2007), and even if waterbugs only periodically prey on cavefish, they could still exhibit strong selection on populations of cavefish. It remains to be investigated whether and to what extent smaller *Belostoma* are supported by dipteran larvae and cavefish (juveniles) and whether the ontogenetic shifts in habitat uncovered in this study are paralleled by a shift in use of trophic resources (in our study, only adult *Belostoma* were analyzed for the stable isotope composition). Shallow habitats with leaf litter, the preferred habitat of small *Belostoma*, also are occupied by snails and dipteran larvae, and juveniles may effectively be foraging on sources rarely exploited by adults.

Our analyses document that waterbugs appear to be capable of completing their entire life cycle within the Cueva del Azufre, as various life stages are present (zygotes, juveniles, and breeding adults). While the present study provides the first basic data on the ecology of *Belostoma* in the sulfidic Cueva del Azufre, further studies are required to better understand the dynamics in the predator-prey relationship of waterbugs and cavefish.

For example, it is as yet unknown how *Belostoma* cope with the toxic effects of hydrogen sulfide, which in the concentrations present in the cave is lethal for most metazoans (Bagarinao, 1992; Tobler et al., 2006). Furthermore, it is unknown if and how well the population of *Belostoma* within the cave is connected to populations in adjacent habitats at the surface. Mollies in the study region have diverged phenotypically and genetically between habitats at the surface and in the cave, although there is no physical barrier that would prevent fish from moving between habitats, indicating ongoing ecological speciation (see Tobler et al., 2008c). Consequently, studies are now required to estimate rates of migration of waterbugs between habitats at the surface and in the cave and to test whether *Belostoma* within the cave also diverged phenotypically and genetically in a manner similar to mollies. Considering that adult *Belostoma* can fly, connectedness between populations in the cave and at the surface may be much larger than that in fish.

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