Sulphide-toxic habitats are not refuges from parasite infections in an extremophile fish

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ABSTRACT

We still have a limited understanding of why animals colonize extreme environments, but one hypothesis (the Pathogen Refuge Hypothesis, PRH) posits that they might represent a safe haven from parasites and other pathogens. We tested this idea by quantifying endoparasite infections in five populations of the neotropical freshwater fish *Poecilia mexicana*, three populations from toxic, H2S-rich habitats and two control populations from nearby rivers and creeks. Counter to predictions derived from the PRH, only *P. mexicana* from toxic habitats harboured heterophyid metacercariae in their hearts. We further uncovered differences in parasite prevalences and abundances between the three toxic habitats. Our results provide evidence against the PRH and add depth to our understanding of life in (and adaptation to) extreme environments.

*Keywords:* ecological speciation, *Poecilia mexicana*, parasitism, hydrogen sulphide, trematode metacercariae, Pathogen Refuge Hypothesis

**1. Introduction**

The colonization of extreme environments is, by definition, associated with costly adaptations to cope with the physical and/or chemical stressors that render these habitats extreme (Townsend et al. 2003). This makes it difficult to explain why organisms would ever actively invade and colonize such habitats, although it is also possible that some ‘extremophiles’ originally entered the extreme environment by accident, became trapped, and then had to ‘adapt or perish’ (Danielopol & Rouch 2005).

The Pathogen Refuge Hypothesis posits that one potential side-benefit of invading extreme environments might come into play if these environments act as a refuge from parasites and other pathogens, which could partially balance out the costs associated with survival (at an early stage) and local adaptation (at a later stage) to the extreme conditions (Kruckberg 1992; Springer et al. 2007; Tobler et al. 2007a). While recent research has shown that extreme environments are not entirely free of parasites (Tinsley 1999; Martinez & Merino 2011; Carlsson et al. 2012; Tobler et al. 2014), several studies have indeed provided evidence for a reduction of at least certain types of parasites in extreme habitats (e.g., oil-pollution and ectoparasites on guppies, *Poecilia reticulata*: Schelkle et al. 2012; pathogens on serpentine flax, *Hesperolinon* spp.: Springer 2009; fish ectoparasites in toxic hydrogen sulphide-rich habitats: Tobler et al. 2007a; high salinity and chytrid fungus infections in eastern dwarf tree frogs, *Litoria fallax*: Stockwell et al. 2015; but see Springer et al. 2007).

Here, we make use of the well-established study system of Mexican livebearing fishes (family Poeciliidae) undergoing population divergence and speciation in toxic hydrogen sulphide (H2S)-rich aquatic habitats (Tobler et al. 2018). Previous research has investigated some aspects of host-parasite interactions in these systems (Tobler et al. 2007a, 2014), and suggested that H2S-rich habitats might indeed act as a refuge from certain ectoparasites (Tobler et al. 2007a), while overall parasite diversity was quite high and individual-level parasite loads were actually higher in H2S compared to non-H2S habitats (Tobler et al. 2014). However, previous studies only focused on some external and internal organ systems, while internal organ systems critical to physiological homeostasis and immediate survival have so far not been screened for parasites. To fill this critical gap of knowledge, we quantified parasitism of internal organs while specifically focussing on the liver, heart and brain of *Poecilia mexicana* from three sulphidic habitats (including an H2S-rich cave) and two non-sulphidic habitats, and we asked if sulphidic habitats might act as a refuge from parasites infecting these organs.

**2. Material and methods**

*2.1. Study system*

Male, female and juvenile Atlantic mollies (*P. mexicana*) were collected in 2014 in the southern Mexican state of Tabasco using seines or dip nets. We collected fish from three sulphidic sites of very similar H2S concentrations (~20 µM; Tobler et al. 2008, 2011): the surface streams El Azufre (EA; latitude: 17.44225, longitude: -92.77447) and La Lluvia (LL; 17.46387, -92.89541), and from chamber V of a sulphidic cave, the Cueva del Azufre (CdA; 17.44234, -92.77542). We also collected them from two non-sulphidic reference sites: the Río Puyacatengo (Puy; 17.47000, -92.89573) and the Río El Azufre, west branch (REA; 17.55634, -93.00762; Fig. 1; for additional habitat descriptions, please see Tobler et al. 2011). Immediately upon capture, fish were anaesthetized with clove oil, and then preserved in 10% formaldehyde solution.

Despite the spatial proximity of CdA and EA, we argue that they constitute independent sampling sites: our previous research has demonstrated strong reproductive isolation between *P. mexicana* populations from both sites (e.g., Plath et al. 2007, 2010; Tobler et al. 2009). They are ecologically different habitats, with EA being a toxic surface creek while CdA represents a toxic subterranean habitat (Plath et al. 2007, 2010; Tobler et al. 2009), and a previous study reported differences in fish parasite communities (Tobler et al. 2014).

*2.2. Data collection*

The body length and weight of all specimens were recorded before they were dissected and all organs as well as body wall musculature were examined for metazoan parasites using standard parasitological techniques (Buchmann 2007). In short, the external body surface and mouth cavity were examined under a Zeiss stereo dissecting microscope. All internal organs, tissues, eyes, and gills were removed, dissected, and examined under a Zeiss Axioskop compound microscope. Any parasites found were noted and counted.

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Figure 1. Overview of the study area with reference cities in grey. Drainages are underlined, and black arrows indicate the location of sulphidic sampling sites, while white arrows indicate the location of non-sulphidic sampling sites. CdA: Cueva del Azufre, chamber V; EA: El Azufre; LL: La Lluvia; Puy: Río Puyacatengo; REA: Río El Azufre, west branch (modified after Plath et al. 2013).

*2.3. Statistical analyses*

First, we compared the prevalence of parasites between sulphidic and non-sulphidic habitats by means of a Fisher’s Exacttest. This was followed by another Fisher’s Exact test to investigate differences in prevalence of parasites between males, females, and juveniles. Both analyses were conducted in IBM SPSS Statistics Version 21.

The prevalence (percentage of fish in each sample infected with a parasite species), mean intensity (mean number of parasites per infected host), and mean abundance (average occurrence of a given parasite species among all members of a particular host population) were calculated according to Bush et al. (1997). We tested for differences in these indices between populations from sulphidic habitats by means of *χ*2-tests(prevalence) and bootstrap two-sample *t*-tests (mean intensity and abundance) using the Quantitative Parasitology 3.0 statistical package (Reiczigel & Rozsa 2005).

**3. Results**

*3.1. Parasite infections*

Examinations of 113 *P. mexicana* from the five habitats revealed no parasites in any organs except for the hearts of fish from sulphidic sites, which harboured infections with trematode metacercarial cysts of the family Heterophyidae. Metacercariae in the heart were found only in the bulbus arteriosus. Intensity of infections ranged from 1 to 33 in each infected fish. In heavily parasitized individuals, the bulbus arteriosus was enlarged, with metacercarial cysts occasionally observed lodged in the confluence with the ventral aorta. Cysts were found either individually within the lumen of the bulbus arteriosus (Fig. 2a) or in clusters held together by host-derived fibrous tissue (Fig. 2b). In non-sulphidic sites, the fish were not infected by any metazoan parasites (Table 1) and consequently, there was a significant difference in parasite prevalence between the two kinds of sites (Fisher’s Exact Test: *P* < 0.001; Fig. 2c).

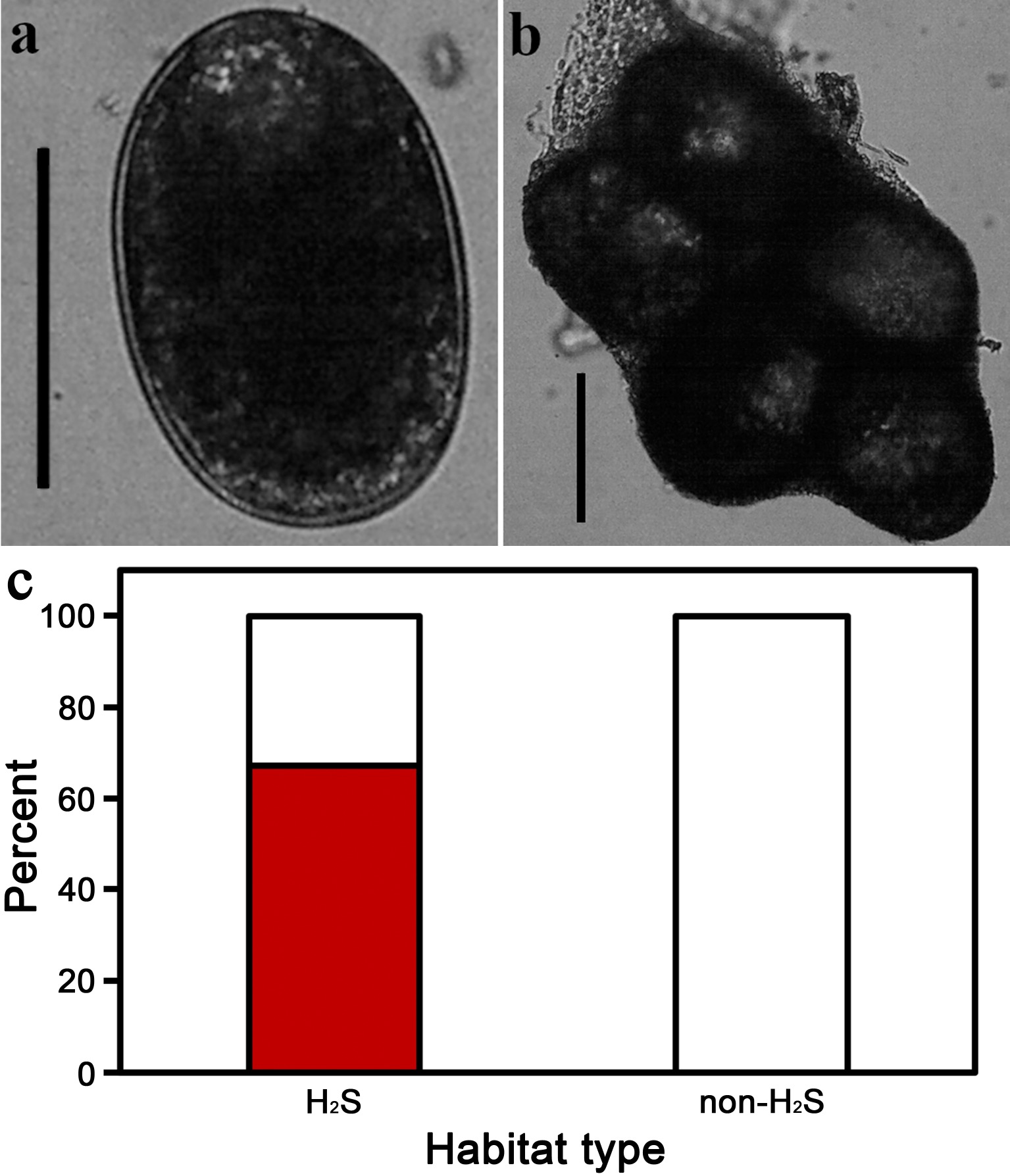


Figure 2. (a) Heterophyid metacercarial cyst isolated from the bulbus arteriosus of *P. mexicana* (scale bar = 100 μm). (b) Clusters of heterophyid metacercarial cysts surrounded by host-derived fibrous tissue from the bulbus arteriosus (scale bar = 100 μm), and (c) parasite prevalence with proportion of infected fish (dark red) and non-infected fish (white) in sulphidic vs. non-sulphidic habitats.

Table 1. Body size and weight of *P. mexicana* from sulphidic and non-sulphidic sites and level of infection with heterophyid metacercariae. Condition factor *K* was calculated as 1,000 × weight [g] / length [mm]3; following Tobler et al. (2006).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Habitat | | *N* | Mean standard length ± s.d. (range) [mm] | Mean weight ± s.d. (range) [g] | Condition factor *K* ± s.d. (range) | Prevalence [%] | Mean intensity ± s.d. | Mean abundance ± s.d. |
| Sulphidic sites | |  |  |  |  |  |  |  |
|  | Cueva del Azufre V | 33 | 23.15 ± 6.00  (12-36) | 0.32 ± 0.19  (0.06-0.80) | 0.025 ± 0.010  (0.016-0.075) | 63.6 | 10.57 ± 10.55 | 6.72 ± 9.81 |
|  | El Azufre | 27 | 23.52 ± 11.22  (11-52) | 0.48 ± 0.64  (0.02-2.85) | 0.026 ± 0.012  (0.014-0.083) | 85.2 | 9.22 ± 8.37 | 7.82 ± 8.39 |
|  | La Lluvia | 13 | 27.92 ± 4.97  (19-36) | 0.54 ± 0.26  (0.16-1.11) | 0.024 ± 0.005  (0.020-0.041) | 38.5 | 5.60 ± 4.78 | 2.15 ± 3.95 |
| Non-sulphidic sites | |  |  |  |  |  |  |  |
|  | Puyacatengo (upstream) | 31 | 27.74 ± 5.20  (19-38) | 0.55 ± 0.30  (0.18-1.37) | 0.024 ± 0.005  (0.020-0.046) | 0 | — | — |
|  | Río El Azufre  (west branch) | 9 | 34.67 ± 13.83  (22-69) | 1.69 ± 2.81  (0.26-9.12) | 0.026 ± 0.002  (0.023-0.028) | 0 | — | —- |

3.2. *Differences in metacercarial infections within sulphidic habitats*

Comparing different age classes and sexes (*N* = 11 juveniles, *N* = 8 males and *N* = 54 females) of fish from sulphidic habitats, we found no significant differences with respect to the prevalence of heterophyid metacercariae (Fisher’s Exact test: *P* = 0.429; Fig. 3a). Comparisons of infections between the three sulphidic sites (interpreted at α = 0.025 to correct for multiple testing) revealed no significant differences between the cave habitat (CdA) and the two surface streams (EA and LL) in metacercarial prevalence (*χ*2 ≤ 3.526, *P ≥* 0.060; Fig. 3b) or mean intensity (*t* ≤ 1.583, *P* ≥ 0.123), although there was a non-significant trend for differences in abundance between CdA and LL (*t*44 = 2.253, *P* = 0.033; Fig. 3c). Nonetheless, metacercarial prevalence and mean abundance, but not mean intensity, were significantly different between the two sulphidic surface stream sites (prevalence: *χ*2 = 9.122, *df* = 1, *P* = 0.003; mean abundance: *t*38 = 2.920, *P* = 0.005; mean intensity: *t*26 = 1.312, *P* = 0.221).

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Figure 3. (a) Parasite prevalence in sulphidic habitats, with infected fish shown in dark red and non-infected fish in white, in juvenile fish and adults of both sexes, and (b) parasite prevalence across the three sulphidic habitats (across sexes and life stages); (c) mean (± 95% CI) parasite abundance across the three sulphidic habitats. CdA: Cueva del Azufre, EA: El Azufre, LL: La Lluvia.

**4. Discussion**

Contrary to our *a priori* prediction, we only found endoparasites in fish from sulphidic habitats, providing strong evidence against the Pathogen Refuge Hypothesis (Kruckeberg 1992; Springer et al. 2007; Tobler et al. 2007a). Surprisingly, we did not discover any parasites in fish from non-sulphidic sites, which could partly be explained by those habitats harbouring a diverse fish community (Riesch et al. 2009), so parasite prevalence in *P. mexicana* from those habitats might be diluted due to the parasites also having additional host species (including several other poeciliids) available (e.g., Civitello et al. 2015). Moreover, predatory fish are usually lacking from sulphidic habitats (e.g., Tobler et al. 2006), but bird predation rates are substantially greater in sulphidic surface than non-sulphidic surface habitats (Riesch et al. 2010), and invertebrate piscivores occur in high densities—and are strong predators of *P. mexicana*—in all sulphidic habitats including the cave (e.g., *Belostoma* sp.: Tobler et al. 2007b; several species of spiders: Horstkotte et al. 2010). Hence, it is possible that predators remove parasitized fish faster in non-sulphidic than sulphidic habitats, and this could help explain the lack of metacercariae in *P. mexicana* from non-sulphidic habitats in our present study. However, we find this scenario less likely.

We further found evidence for some differences between sulphidic habitats, with LL being different from CdA and EA. Since all three sites have very similar H2S-levels (Tobler et al. 2008, 2011), this might reflect some, as yet unidentified, differences between the two different drainages in which these sites are situated (e.g., encounter rates with definitive hosts, see below).

Heterophyid metacercariae are a dominant group of trematodes parasitizing fish in Mexico (Scholz et al. 2001). Adult parasites typically live in the intestines of fish-eating mammals and birds. Eggs produced by sexually-mature worms are passed out with faeces and can be ingested by aquatic snails, in which asexual development, from sporocysts to redia, occurs. The redia eventually produce cercariae—a free-living stage—which emerge from the snail to locate and infect a fish host where it encysts into metacercariae, a largely dormant stage, waiting for the chance to complete its development if the fish is eaten by a piscivorous mammal or bird (Scholz et al. 2001). High intensity infections of heterophyid metacercariae in the bulbous arteriosus of fish are known to cause mechanical blockage of the heart, inhibiting normal blood flow and decreasing swimming endurance (Coleman 1993). Heavily parasitized fish are more quickly exhausted and consequently cease swimming, thereby increasing the likelihood of predation by a piscivorous vertebrate and promoting the continued maintenance of the parasite’s life cycle.

It is not known if the metacercariae recovered in the present study are the result of complete cycling of the parasite’s life history within sulphidic habitats or if the fish infections simply represent a ‘dead end’. However, while the exact species are currently unknown, snails are indeed available in all sulphidic habitats, including the sulphidic cave (Roach et al. 2011). Similarly, piscivorous birds are particularly common around sulphidic surface habitats (Riesch et al. 2010). While birds are absent from the sulphidic cave, tracks and scat found in the cave during our field work (RR, JJ, MP and LAR, personal observation) suggest that nocturnal piscivorous mammals, such as racoons (*Procyon lotor*) or the water opossum (*Chironectes minimus argyrodytes*), indeed venture into the cave on a fairly regular basis, and could thus act as definitive hosts for this parasite.

A previous study focusing on another trematode (*Uvulifer* sp.), encysting in the skin, found their prevalence on *P. mexicana* to be highly reduced in sulphidic habitats and to be absent in the sulphidic cave (Tobler et al. 2007a). Given the results from our current study, this is somewhat surprising since they would largely rely on similar hosts as the hetrophyid trematodes we discovered in the hearts of *P. mexicana*, and both have free-living cercaria stages. These differences in prevalence could be explained by two not mutually exclusive hypotheses. First, the location of infection within *P. mexicana* coupled with the extreme toxicity of hydrogen sulphide (Tobler et al. 2018): *Uvulifer* sp. burrow into the skin and then encyst relatively close to the body-water interface (Tobler et al. 2007a), while the hetrophyid trematodes we report on here encyst in the heart. For trematodes in the heart, the fish’s body acts as a shield against H2S-toxicity. *Poecilia mexicana* has evolved several genetic modifications to detoxify H2S entering the body or physiologically cope with H2S-toxicity (e.g., *cox*-gene evolution; Pfenninger et al. 2014). *Uvulifer* metacercariae, on the other hand, would only have a relatively thin layer of fish tissue protecting them from direct long-term exposure to H2S. Second,adult *Uvulifer* sp. typically live in the intestines of kingfishers (their preferred definitive host; Hoffman 1999), and piscivorous birds do not frequent the Cueva del Azufre.

Low quality environments can have varying effects on the occurrence of trematodes in fish and molluscs (Lafferty 1997; Morley et al. 2003, 2006; Vidal-Martinez et al. 2010), which may be the result of the exposure of free-living stages to environmental stressors. Unfortunately, the effects of sulphidic environments on trematodes are poorly understood, and so we currently do not know how the free-swimming cercariae of both trematodes cope with short-term H2S toxicity. Future genomic studies including these parasites would be of great interest, and the evolution of, for instance, metabolic pathways in *P. mexicana* (see Kelley et al. 2016) and heterophyid parasites could be another example of host-parasite coevolution.

Regarding the Pathogen Refuge Hypothesis (Kruckberg 1992; Springer et al. 2007; Tobler et al. 2007a), the picture is now becoming more complex. While previous studies often found support for this hypothesis with respect to certain extreme environments and certain parasite species (Tobler et al. 2007a; Springer 2009; Schelkle et al. 2012; Stockwell et al. 2015), our study adds to the literature that found evidence against the hypothesis (see also Springer et al. 2007). Clearly, whether or not the colonization of extreme habitats is a result of patterns congruent with the Pathogen Refuge Hypothesis is system- and pathogen-specific. Nonetheless, our study demonstrates that selective regimes stemming from pathogens and parasites in extreme environments are more complex than often assumed.

**Data availability**

Data underlying this study can be found in the Supplementary material, Table S1.

**Author contributions**

JJ and LAR collected the fish; RR conceived the idea for the study; NM collected the data; NM and RR analysed data; RR and MP drafted the first manuscript version, all authors contributed to revisions, gave final approval for publication, and agree to be held accountable for the content herein.

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**Declaration of competing interests**

None.

**Ethics**

The study complies with the current laws on animal experimentation of the United Kingdom; no specific ethics approval was needed.

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