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# Host-parasite interactions in perpetual darkness: Macroparasite diversity in the cavefish *Astyanax mexicanus*

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## ABSTRACT

*Astyanax mexicanus* has repeatedly colonized cave environments, displaying evolutionary parallelisms in many troglobitic traits. Despite being a model system for the study of adaptation to life in perpetual darkness, the parasites that infect cavefish are practically unknown. In this study, we investigated the macroparasite communities in 18 cavefish populations from independent lineages and compared them with the parasite diversity found in their sister surface fish populations, with the aim of better understanding the role that parasites play in the colonization of new environments. Within the cavefish populations, we identified 13 parasite taxa, including a subset of 10 of the 27 parasite taxa known for the surface populations. Parasites infecting the cavefish belong to five taxonomic groups, including trematodes, monogeneans, nematodes, copepods, and acari. Monogeneans are the most dominant group, found in 14 caves. The macroparasites include species with direct life cycles and trophic transmission, including invasive species. Surprisingly, paired comparisons indicate higher parasite richness in the cavefish than in the surface fish. Spatial variation in parasite composition across the caves suggests historical and geographical contingencies in the host-parasite colonization process and potential evolution of local adaptations. This base-line data on parasite diversity in cavefish populations of *A. mexicanus* provides a foundation to explore the role of divergent parasite infections under contrasting ecological pressures (cave vs. surface environments) in the evolution of cave adaptive traits.

**Keywords:** Adaptation; Metazoan parasites; Niche change; Parasite assemblage; Prevalence

## INTRODUCTION

Hosts and parasites are engaged in complex interactions of constant reciprocal adaptation, imposing strong selective forces on each other, in some cases, influencing their evolutionary trajectories (e.g., Bush et al., 2019). During the colonization of a new environment, hosts may lose parasites (enemy release hypothesis; Colautti et al., 2004) and maintain a subset of their original diversity, generating new parasite assemblages (Hoberg et al., 2012). Changes in these dynamics can alter host-parasite interactions (Best et al., 2017; Wolinska et al., 2008), and produce rapid adaptations (Eizaguirre et al., 2012). Parasite selective pressures have implications for natural and sexual selection, for example, behavioral modulation could change host habitat selection to avoid or promote parasite infection (Demandt et al., 2018; Eizaguirre & Lenz, 2010; Jolles et al., 2020; Mikheev et al., 2013), or parasite-influenced assortative mating could directly or indirectly influence mate choice (Milinski, 2014).

Differences in parasite infections are commonly associated with trophic disparity or environmental filters leading to local adaptations (Hablützel et al., 2017; Karvonen & Seehausen, 2012; Wegner et al., 2003). For instance, hosts may display defensive mechanisms to resist unique parasitic infections in the ecosystem (Eizaguirre et al., 2011, 2012), which could result in positive or negative fitness effects on the residents and/or immigrants and hybrids (Karvonen & Seehausen,



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2012), even limiting genetic flow between populations (Erin et al., 2019). The complexity of the transmission strategies of parasites (i.e., direct or indirect life cycles) and host life history, using only aquatic hosts (autogenic life cycle) or cycling through both aquatic and terrestrial hosts (allogenic life cycle), are crucial factors influencing successful dispersal and colonization (Criscione & Blouin, 2004). Trophically-transmitted species (indirect life cycle) rely heavily on suitable hosts to complete their life-cycle. However, in ecosystems with limited nutrient availability, the opportunities for host-shifts are reduced. For parasites with direct life cycles, the host represents the environment (Lymbery, 2015), acting as a buffer against challenging external conditions. Therefore, the trade-off with the environment can shape the evolution of host-parasite interactions (Brunner & Eizaguirre, 2016; Brunner et al., 2017).

*Astyanax mexicanus* displays an extraordinary evolvability, enabling it to repeatedly colonize cave environments characterized by low food availability and perpetual darkness. Across different subterranean rivers, *A. mexicanus* has independently evolved phenotypes typical of troglobitic organisms, such as the absence of eyes and pigmentation. Currently, there are 35 known cave populations distributed along three mountain ranges in Northeastern Mexico (Espinasa et al., 2018, 2020; Miranda-Gamboa et al., 2023; Proudlove, 2019), acting as biogeographic barriers for the fish divergence and delimiting their phylogeographic patterns (Garduño-Sánchez et al., 2022; Herman et al., 2018). The cave and surface populations represent two independent lineages, corresponding to independent waves of recent colonization (Herman et al., 2018; Ornelas-García et al., 2008; Strecker et al., 2004), referred to as “Lineage 1” and “Lineage 2” (Garduño et al., 2022; Moran et al., 2023). Notably, *A. mexicanus* serves as a fascinating model, as cavefish can still interbreed with surface-dwelling fish, leading to the existence of natural hybrid populations (Moran et al., 2022) and providing the opportunity to study the inheritance of cave-adaptive traits. Cavefish have undergone a range of adaptations in response to perpetual darkness, encompassing morphological, behavioral, and physiological modifications (Gross & Powers, 2020; Hyacinthe et al., 2019; Jeffery, 2020; Kowalko, 2020; Wilkens & Strecker, 2017). These adaptations have led to various evolutionary outcomes, including the enhancement of sensory structures to navigate in the dark (Yoshizawa et al., 2010) and metabolic changes to resist prolonged periods of starvation (Riddle et al., 2018; Xiong et al., 2022).

Despite the progress and establishment of *A. mexicanus* as a study model, little is known about its natural interactions. Studies on wild populations have addressed aspects of its diet (Espinasa et al., 2017), microbiome (Ornelas-García et al., 2018), circadian rhythm (Beale et al., 2013), olfactory responses (Blin et al., 2020), and parasites (Peuß et al., 2020; Santacruz et al., 2020b), covering only a few caves. Surface-dwelling *A. mexicanus* harbors a diverse range of macroparasites, forming ancient and highly host-specific parasite associations, primarily with helminths such as trematodes, monogeneans, acanthocephalans, and nematodes (Pérez-Ponce de León & Choudhury, 2005; Santacruz et al., 2020a, 2020b). Previous studies have also shown that parasites can exert selective pressures on host adaptive traits (Binning et al., 2017; Hoste, 2001; Nadler et al., 2021), thereby maintaining or eroding differences in host contact hybridization zones (Theodosopoulos et al., 2019), or fueling host divergence and speciation (Karvonen & Seehausen, 2012). Therefore, to fully understand the mechanisms operating in cavefish adaptation, it is crucial to investigate their biotic interactions, including potential host-parasite interactions occurring within the cave systems.

Caves are an ideal ecological setting to test how repeated colonization of novel habitats impacts host-parasite interactions. This first requires a comprehensive understanding of parasite diversity. Thus, in the current study, we aimed to: (1) characterize the macroparasite species diversity in 18 cave and six surface populations of *A. mexicanus*, corresponding to independent colonization lineages, (2) test the spatial rearrangement of parasite assemblages under contrasting ecological pressures (cave and surface rivers), and (3) determine whether the same parasite lineages are shared across surface and cavefish populations.

## MATERIALS AND METHODS

### Sample collection

Fish samples were collected from 2015 to 2021 in populations in three geographical areas, i.e., Sierra de Micos (Colmena), Sierra de El Abra, and Sierra de Guatemala, consisting of 18 cavefish populations and six nearby surface fish populations (Table 1). Permission for the collection of cave specimens was obtained from the relevant authorities (SEMARNAT SGPA/DGVS/2438/15-16, SGPA/DGVS/05389/17, and SGPA/DGVS/1893/19). Upon capture, most fish were immediately euthanized in cold water with an overdose of tricaine (MS-222) and preserved in absolute ethanol for later DNA extraction and parasitological screening. Other fish were scanned immediately after euthanization on the same sampling day, with voucher specimens preserved in ethanol. After collection, certain fish specimens were transported to the laboratory, where they were kept in isolation for parasite screening several days later. Fish euthanization was carried out in strict accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2020 edition (https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf). The fish were deposited in Colección Nacional de Peces, Instituto de Biología, National Autonomous University of Mexico (UNAM). The parasitological material was deposited in the National Collections of the UNAM, Mexico City: Colección Nacional de Helmintos (CNHE) and Colección Nacional de Ácaros (CNAC).

### Parasite load

Fish analyzed on the same day as sampling or stored in alcohol immediately after collection showed no differences in parasitic loads, while fish analyzed days later showed some changes in parasitic abundance. Parasitological screening was performed under a stereomicroscope (Leica Zoom 2000, Germany). Each fish was fully screened to collect macroparasites (i.e., helminths, crustaceans, and mites). The scan included the skin, gills, mouth, and external eyes for ectoparasites, and internal eyes, heart, gonads, liver, gastrointestinal tract, gall bladder, spleen, and abdominal cavity for endoparasites. Parasites were removed from the host tissues with surgical needles, washed in 6.5% saline solution, counted, and stored in molecular biology-grade ethanol.

**Table 1 Characteristics of fish populations sampled from cave and surface populations of *Astyanax mexicanus*: lineage, geographic region, population, number of fish individuals, fish screened in lab or field, and fish screened completely or only the gills**

| Lineage 1 (L1) or Lineage 2 (L2) | Geographic region | Population name | *n* | Field (F) or Lab (L) | Complete (C) or gills (G) |
|---|---|---|---|---|---|
| L1 | Micos | Micos | 39 | F | C |
| L1 | Guatemala | Molino | 20 | F | C/G |
| L1 | Guatemala | Escondido | 5 | F | C |
| L1 | Guatemala | Vázquez | 5 | F/L | C |
| L1 | Guatemala | Caballo Moro | 5 | F | C |
| L2 | Abra | Pachón | 19 | F | C |
| L2 | Abra | Yerbániz | 2 | L | G |
| L2 | Abra | Japónes | 4 | F | C |
| L2 | Abra | Tigre | 5 | L | C/G |
| L2 | Abra | Sabinos | 29 | F | C/G |
| L2 | Abra | Arroyo | 21 | F | C/G |
| L2 | Abra | Tinaja | 31 | F/L | C/G |
| L2 | Abra | Montecillos | 12 | F/L | C |
| L2 | Abra | Jos | 9 | F | C |
| L2 | Abra | Palma Seca | 18 | L | C/G |
| L2 | Abra | Pichijumo | 5 | F/L | C |
| L2 | Abra | Chica | 25 | F | C |
| L2 | Abra | Toro | 2 | F | C |
| L1 | Abra | Puente Guémez | 12 | F | C |
| L1 | Abra | Guayalejo | 10 | F | C |
| L2 | Abra | Otates | 9 | F | C |
| L1 | Abra | Nacimiento Mante | 3 | F | C |
| L1 | Abra | Florido | 3 | F | C |
| L1 | Micos | Santa Anita | 16 | F | C |

**Taxonomic identification**

The identification of parasites followed standard morphological techniques for each group of parasites. Trematodes and monogeneans were stained with Gomori's trichrome or Mayer's paracarmine, while nematodes, acari, and copepods were cleared in alcohol-glycerin (1:1) solution. Monogeneans were excised, with the anterior or posterior body end preserved in ethanol and the remaining half partially enzymatically digested to preserve sclerotized structures: haptor or male copulatory organ (MCO). They were then mounted in Gray-Weiss solution as permanent slides. Parasites were observed and photographed under a motorized inverted microscope (Olympus IX81, Japan) equipped with differential interference contrast (DIC) optics. The ultrastructure of certain parasites was assessed by scanning electron microscopy (SEM) using a 10 kV Hitachi Stereoscan Model SU1510 microscope following Santacruz et al. (2020b). All parasites were identified at the highest possible taxonomic level. All literature records of helminths in *A. mexicanus* were compiled from their original sources (e.g., Salgado-Maldonado et al., 2004; Salgado-Maldonado, 2006; Santacruz, 2013; Santacruz et al., 2020a, 2020b), in addition to new records generated in this work. New species will be described in separate studies.

**Molecular analysis**

To further investigate parasite conspecificity across surface and cavefish populations, specimens fixed in 100% ethanol were individually sequenced. The molecular markers used were the mitochondrial gene cytochrome c oxidase subunit 1 (*COI*) and nuclear genes *18S* and *28S*, depending on the genetic library available for each parasite group (Supplementary Tables S1, S2). Phylogenetic position was then determined using maximum likelihood with the IQ-TREE v1.6.2 web platform (http://iqtree.cibiv.univie.ac.at/) (Nguyen et al., 2015). Genetic distances were estimated as uncorrected *P*-distances in MEGA v7 (Kumar et al., 2018).

**Data analysis**

We calculated parasite species richness as the number of parasite taxa identified for each host; prevalence as the proportion of infected hosts by a given parasite species; abundance as the number of conspecific parasites in the host population; and intensity as the number of conspecific parasites in the population of infected hosts (Bush et al., 1997; Rózsa et al., 2000). Using a Wilcoxon test, we conducted paired comparisons of the parasite richness between two caves and their respective lineage's surface populations: i.e., Micos Cave vs. Santa Anita River for Lineage 1, and Arroyo Cave vs. Otates River for Lineage 2.

To evaluate the similarity of parasite assemblages, we used the diversity values of the infracommunity, defined as all parasite species in a host at one point in time (Bush et al., 1997). With the abundance values of each parasite species, we used the Bray-Curtis dissimilarity metric as a distance measure and performed non-metric multidimensional scaling (NMDS) using the "metaMDS" function in the R package "vegan v1.13-1" (Oksanen et al., 2019). In the NMDS each point represents an infracommunity. The more similar the infracommunities are, the closer the points are to each other. We used locality, habitat (cave or surface), and geographic region as factors in the analysis. Figures were produced using the R package "ggplot2" (Valero-Mora, 2010). For analysis, we excluded individuals with incomplete data (only gill parasite data) and/or individuals kept in the laboratory before parasite screening. To evaluate the extent to which these differences may be explained by locality, habitat, or geographic region, we performed permutational multivariate analysis of variance (PERMANOVA).

## RESULTS

### Sampling

In total, we sampled 309 fish, including 256 cavefish and 53 surface fish, from 18 cave and six surface populations, respectively (Table 1; Figure 1). We categorized the samples according to their geographical regions (Sierra de Guatemala, Sierra de El Abra, and Sierra de la Colmena (Micos)) and lineages ("Lineage 1" and "Lineage 2").

### Parasite diversity

We recovered 13 macroparasite taxa from the cavefish populations, including endoparasites and ectoparasites with contrasting transmission strategies and in different life stages (larvae and adults). The taxa belong to five taxonomic groups: i.e., trematodes, monogeneans, nematodes, copepods, and acari (Table 2; Figure 1). The most common parasites were monogeneans, found in 14 cavefish populations, followed by nematodes in eight, trematodes in five, acari in two, and

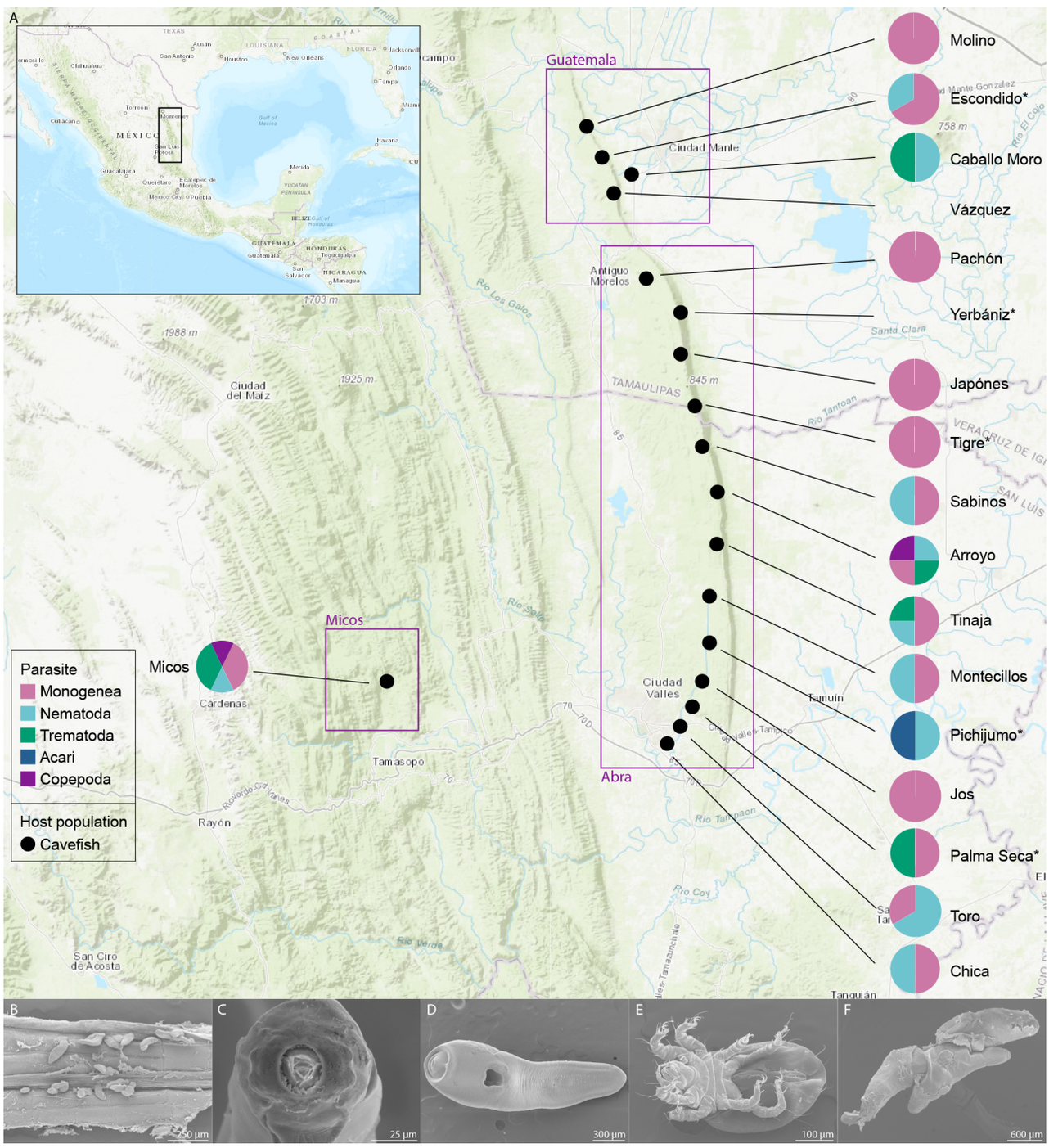


**Figure 1 Geographical location of caves sampled in Northeastern México**

A: Pie charts show proportions of parasite species by taxonomic group. Data from Jos and Molino caves are only available for gill parasites. Asterisk indicates fish populations kept in the laboratory before parasite screening. B–F: Photomicrographs of representative parasites in cavefish. B: *Cacatuocotyle* cf. *chajuli* monogeneans attached to cavefish anal fin; C: Anterior end of nematode *Procamallanus neocaballeroi* Lineage 1; D: Trematode *Clinostomum* sp.; E: Oribatid mite; F: Copepod *Lernaea cyprinacea.*

**Table 2 Inventory and life history traits of parasites infecting surface fish and cavefish populations of *A. mexicanus* across its geographical distribution**

| Parasite group | Parasite | Global | | Site of infection | Stage | Life cycle | | Transmission pathway |
|---|---|---|---|---|---|---|---|---|
| | | SF | CF | | | (D/I) | Au/Allo | |
| **Trematoda** | *Ascocotyle tenuicollis* | | | Gills | L | I | Allo | Snail (first), fish (second), bird (definitive) (1) |
| | *Centrocestus formosanus* * | | | Gills | L | I | Allo | Snail (first), fish/amphibian (second), bird/mammal (definitive) (2) |
| | *Clinostomum sp.* | | | Skin | L | I | Allo | Mollusc (first), fish (second), bird (definitive) (3) |
| | *Creptotrematina aguirrepequeñoi* | | | Gut | A | − | Au | − |
| | *Genarchella astyanactis* | | | Gut | A | I | Au | Snail (first), copepod (second), fish (definitive) (4) |
| | *Diplostomum* sp. | | | Skin & body cavity | L | I | Allo | Mollusc/fish (first), fish (second), bird (definitive) (5) |
| | *Magnivitellinum simplex* | | | gut | A | − | Au | Snail (first), Diptera/Ephemeroptera (second), fish (definitive) (6) |
| | *Prostenhystera obesa* | | | Gall bladder | A | − | Au | − |
| | Trematoda gen. sp. 1 | | | Gut | A | − | Au | − |
| | Trematoda gen. sp. 2 | | | Gut | A | − | Au | − |
| | *Wallinia mexicana* | | | Gut | A | − | Au | − |
| **Cestoda** | *Schyzocotyle acheilognathi* * | | | Gut | A | − | Au | Copepod (first), fish (definitive) (7) |
| **Monogenea** | *Anacanthocotyle anacanthocotyle* | | | Gills | A | D | Au | Direct |
| | *Cacatuocotyle* cf. *chajuli* | | | Skin | A | D | Au | Direct |
| | *Characithecium* cf. *costaricensis* | | | Gills | A | D | Au | Direct |
| | *Gyrodactylus* sp. | | | Skin | A | D | Au | Direct |
| | *Microcotyle* sp. | | | Gills | A | D | Au | Direct |
| | *Urocleroides* sp. | | | Gills | A | D | Au | Direct |
| | *Urocleidoides strombicirrus* | | | Gills | A | D | Au | Direct |
| **Nematoda** | *Contracaecum* sp. | | | Mesentery | L | I | Allo | Copepod (first), fish (second), bird (definitive) (8) |
| | *Eustrongylides* sp. | | | Mesentery | L | I | − | − |
| | *Hystherothylacium* sp. | | | Mesentery | L | I | − | − |
| | Pharyngodonidae gen. sp. | | | – | – | – | – | – |
| | *Procamallanus neocaballeroi* Lineage 1 | | | Gut | A, L | I | Au | Copepod (first), fish (definitive) (9) |
| | *Procamallanus neocaballeroi* Lineage 2 | | | Gut | A, L | I | Au | Copepod (first), fish (definitive) (9) |
| | *Rhabdochona mexicana* Lineage 1 | | | Gut | A, L | I | Au | Copepod/Ephemeroptera (first), fish (definitive) (10) |
| | *Rhabdochona mexicana* Lineage 3 | | | Gut | A, L | I | Au | Copepod/Ephemeroptera (first), fish (definitive) (10) |
| | *Spiroxys* sp. | | | Mesentery | L | I | Allo | Copepod (first), fish (second), amphibian/reptile (definitive) (11) |
| **Copepoda** | *Lernaea cyprinacea* * | | | Skin | A | D | Au | Direct |
| **Acari** | Oribatida gen. sp. | | | Skin & gills | A | D | Au | Direct (12) |

Gray cells indicate positive records in cavefish (CF) and/or surface fish (SF) populations. Life stage of parasites found in fish as larvae (L) or adult (A), type of life cycle: direct (D) or indirect (I); and autogenic (Au) or allogenic (Allo) are indicated. Asterisk indicates invasive species. (1) De Núñez (2001); (2) Pinto et al. (2018); (3) Dias et al. (2003); (4) Ditrich et al. (1997); (5) Field & Irwin (1995); (6) Davies et al. (2021); (7) Marcogliese & Esch (1989); (8) Køie & Fagerholm (1995); (9) Moravec & Vargas-Vázquez (1996); (10) Moravec (1976); (11) Hasegawa & Otsuru (1978); (12) Olmeda et al. (2011).

copepods in one. No parasites were found in the Yerbaniz and Vázquez caves. Except for an acari harbored by hosts in the Pichijumo and Jos caves, and unidentified trematodes from the Palma Seca and Caballo Moro caves, all parasites are a subset of the 27 taxa or lineages found across the distribution range of surface fish populations (Table 2). Five parasite taxa are shared in four or more caves: monogeneans *Cacatuocotyle* cf. *chajuli* Mendoza-Franco, Caspeta-Mandujano & Salgado-Maldonado, 2012 and *Characithecium* cf. *costaricensis* Mendoza-Franco, Reina, & Torchin, 2009; nematodes corresponding to Lineage 1 (*sensu* Santacruz et al., 2020b) of *Procamallanus neocaballeroi* Caballero-Deloya, 1977 and *Spiroxys* sp.; and trematode *Genarchella astyanactis* Watson, 1976. One invasive species, the anchor worm (*Lernaea cyprinacea* Linnaeus, 1758), was found in a host from Micos Cave.

The genetic lineages of the parasites are shared across the cave and surface populations. The sequence data of some species were deposited in GenBank under accession numbers: *C.* cf. *chajuli, 28S* (OQ888696–99) and *COI* (OQ873440–43); *C.* cf. *costaricensis*, *28S* (OQ888690–95) and *COI* (OQ884019–22); *Spiroxys* sp., *COI* (OQ884015–18);

and *G. astyanactis COI* (OQ873428).

**Infection patterns**

Per-cavefish parasite richness varied between 0–4 taxa, with a mean of 0.93 taxa per host (*SD*=0.95, *n*=251) and a maximum found in individuals from Micos Cave. Parasite species richness in surface populations ranged between 0–3 taxa, with a mean of 0.72 (*SD*=1.18, *n*=56) (Supplementary Table S3; Figure 2). The prevalence, abundance, and intensity of each parasite taxa were calculated, excluding fish kept at the laboratory before screening or incompletely screened for parasites (e.g., only gills). The dataset contained 183 cavefish individuals, 115 (62.8%) of which were infected by at least one parasite. The parasite with the highest prevalence (100%) was the gill monogenean *C.* cf. *costaricensis* in the Pachón and Toro caves, followed by the nematode *Spiroxys* sp. (81%) in Micos Cave (Figure 3). The intensity and abundance of infection were heterogenous for all parasite taxa (Supplementary Table S3). The greatest abundance and intensity for a single parasite species was displayed by *C.* cf. *chajuli* infecting fish from Toro Cave, with 55–87 monogeneans in a single fish.

The paired comparisons between cave and surface hosts showed differences in infection profiles. The two cavefish populations analyzed showed higher parasite species richness than their sister surface populations from the same lineage (Figure 4). Parasite richness was significantly higher in Micos Cave vs. Santa Anita River (Wilcoxon test, *P<0.001*), with a total of nine parasite taxa in the cave population and a subset of two of the nine taxa in the Santa Anita surface population. We also found significant differences between Arroyo Cave vs. Otates River (Wilcoxon test, *P*=0.03). *Spiroxys* sp. was the sole species shared at both sites, and the Otates River population harbored the trematode *Prostenhystera obesa* (Diesing, 1850), which was not found in the other cave population.

**Spatial variation in parasite communities**

We analyzed 125 infracommunities, 11 from cavefish and six from surface fish populations. NDMS analysis showed that the parasite communities were differentiated by population (PERMANOVA, *P*=0.001); individuals from Micos Cave were the most differentiated (Figure 5A). Habitat (cave or surface) also had a significant effect on parasite composition (PERMANOVA, *P*=0.002) (Figure 5B). The strongest clusters were based on geographic region (PERMANOVA, *P*=0.001) (Figure 5C).

## DISCUSSION

The colonization of a new environment involves changes in the host-parasite interaction dynamics. Many parasites colonize new habitats through host-mediated dispersal, which involves a trade-off between life-history and the evolutionary pressures acting on dispersal traits (Perkins et al., 2013). Hence, during colonization the host may lose parasites generating a mosaic of parasite assemblages across different landscapes (Hoberg et al., 2012). Here, we explored the parasite diversity in cavefish and surface fish populations. Our study showed that parasites are very common in caves,

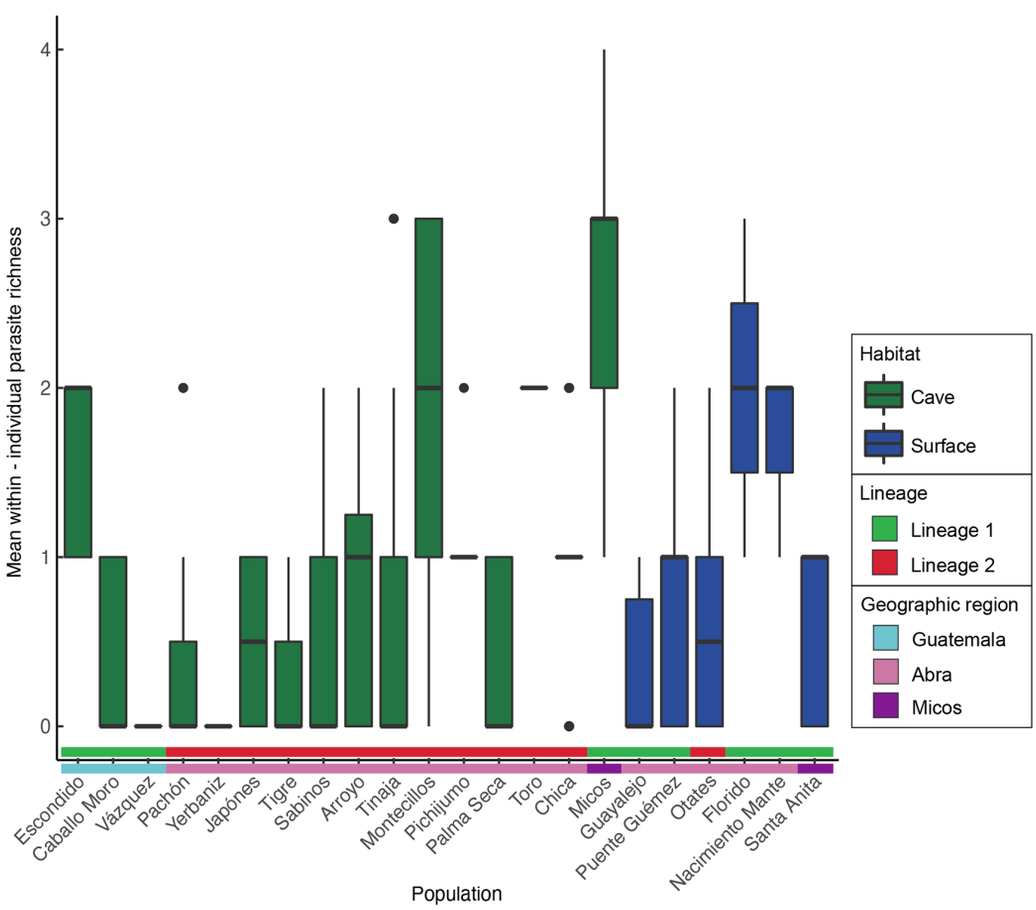


**Figure 2 Mean individual parasite richness**

Colors represent habitat, geographic region, and lineage classification.

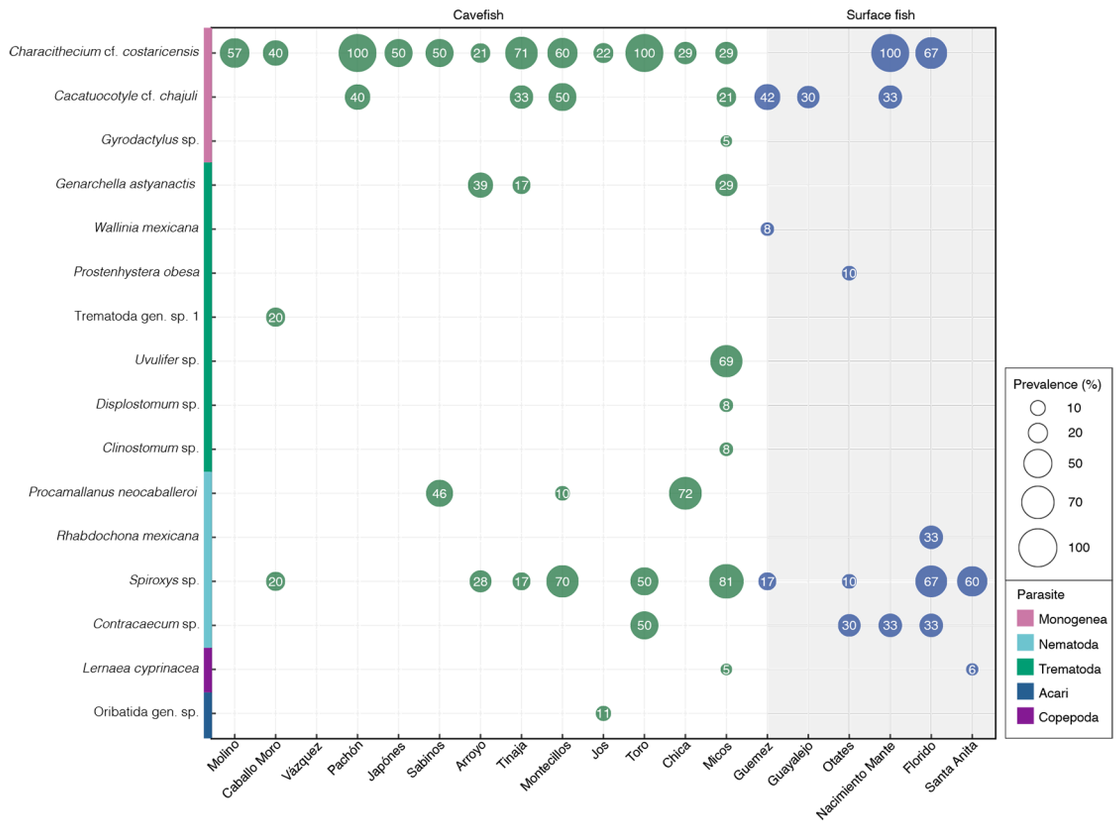


**Figure 3 Parasite prevalence in cavefish and surface fish**

Parasite prevalence varies among host populations and habitat. Sampled surface fish populations are shaded in gray.

forming heterogeneous parasite communities across cavefish populations, suggesting that parasites have managed to colonize these environments with their hosts, and persist after niche change.

In the studied cavefish populations, we identified 13 parasite taxa from distantly related taxonomic groups. Across their geographical distribution, our data suggest a high diversity of parasites in surface fish compared to other fish species in the same area. The cavefish harbors a subset of the total parasite diversity known for surface fish, sharing 10 of the 27 parasite taxa.

The most common cavefish parasites belong to the "core parasite fauna" of Middle American characids (Pérez-Ponce de León & Choudhury, 2005). Here, based on their prevalence and abundance, we propose a "cavefish core parasite fauna", including trematode *G. astyanactis*, monogeneans *C.* cf. *chajuli* and *C.* cf. *costaricensis*, and nematodes *P. neocaballeroi* and *Spiroxys* sp. The presence of these parasites in four or more caves suggests a moderate deterministic pattern: intrinsic traits of the parasites may enable them to elude the same ecological filters, thus allowing repeated colonization of the caves.

Due to their direct transmission strategy requiring only the fish host to complete their life-cycle, monogeneans are the most likely candidates for co-invading the caves alongside with their hosts. Indeed, in our study the monogeneans have been successful colonizers, since they were found in 14 of the 18 caves analyzed. The infrequent presence of monogeneans in surface populations suggests the occurrence of repeated cave invasions, increasing the probability of infected individuals entering the caves and facilitating the establishment of cavefish-monogenean interactions.

In contrast, the remaining members of the "cavefish core parasite fauna" display a trophic transmission strategy; some of them mature and reproduce in the fish whereas others require the fish to be eaten by a predator for the parasite to complete its life-cycle. This scenario implies that other organisms suitable as intermediate hosts also colonized the caves, giving rise to interaction networks occurring in perpetual darkness. For *G. astyanactis* and *P. neocaballeroi*, both infect copepods that are eaten by the fish (Ditrich et al., 1997; Moravec & Vargas-Vázquez, 1996). Instead, *Spiroxys* sp. found as a larva in fish, can mature in amphibians and reptiles (e.g. Li et al., 2014). That is, trophically-transmitted parasites are a good proxy for host diet and host predators predictions (Johansen et al., 2019; Leung & Koprivnikar, 2019). In Pachón cavefish, the diet of non-adult fish is reported to include copepods, ostracods, and isopods (Espinasa et al., 2017), similar to our observations in adult cavefish from different caves (Santacruz, pers. obs.). Regarding predators, their numbers are proposed to be significantly reduced in caves, attributed to cavefish behaviors such as vibration attraction behavior (VAB) and loss of schooling (Kowalko et al., 2013; Yoshizawa et al., 2010). Therefore, one possible alternative is that immature *Spiroxys* sp. somehow enter the caves, leading to a dead-end for the parasite.

The presence of the anchor worm *L. cyprinacea* in cavefish from Micos Cave is noteworthy. Considered an invasive species (Narciso et al., 2019; Zhu et al., 2020), this copepod can cause intense inflammation at the site of attachment, leading to secondary infections (Salinas et al., 2019). The parasite has been usually co-introduced with carp across the world (Steckler & Yanong, 2012). Although no carp were

found in the studied caves, the parasites may have entered during flooding events with already infected surface fish. Another intriguing association that requires further investigation is the infection of mites, not yet taxonomically determined, in the gills and skin of fish from the Pichijumo and Jos caves. While this specific parasite has not been recorded in surface fish, it is worth noting that oribatid mites, in general, have rarely been reported as parasites (Olmeda et al., 2011; Santacruz et al., 2022).

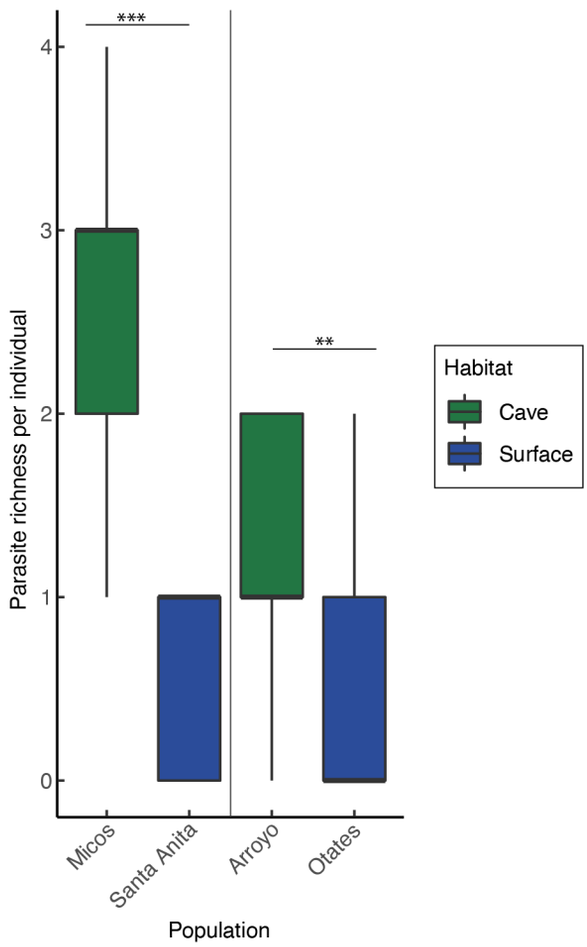


**Figure 4 Per-fish parasite richness varies significantly between paired cave and surface populations**

The heterogeneous nature of parasite communities across caves can be attributed to different scale-specific effects (e.g., host immunity, diet, and sex) (see Bolnick et al., 2020a, 2020b; Poulin & Valtonen, 2002). Numerous studies have shown that exposure to different parasites may result in local adaptations; for instance, differences in resistance or susceptibility in sticklebacks as a result of a parasite-mediated selection on the immune response and/or the mate choice (Eizaguirre et al., 2012; Milinski, 2014; Scharsack et al., 2007; Stutz et al., 2014, 2015). In our paired comparisons between cave and surface parasite infections, we uncovered differences between the two environments, with a tendency for higher levels of infection and aggregation within the caves, and no evidence of parallel infection levels in the same host ecomorphotype. This difference could lead to divergence in the host immune response across cave populations. For instance, the Pachón Cave population exhibits a more sensitive immune response than certain surface fish populations (Peuß et al., 2020), confirming that local adaptations may partially explain why cavefish are more parasitized in particular populations. Additionally, cavefish populations lack some ecological pressures, including predation or interspecific competition, which may allow individuals to tolerate greater parasitic loads. Moreover, since the caves show contrasting abiotic variations compared to those of the surface rivers (Ornelas-García et al., 2018), it leads to the question of how such abiotic differences are shaping host-parasite interactions. For instance, water temperature preferences in cavefish (see Tabin et al., 2018) have been linked to contrasting parasite infections in other fish species (also see Karvonen et al., 2013).

Genetic analysis of the parasite taxa examined revealed shared lineages between the cave and surface populations, which may have multiple possible explanations. For instance, it might be possible that niche change has not yet promoted the parasite divergence, as the parasites may have recently entered the caves, resembling the recent dispersion of their

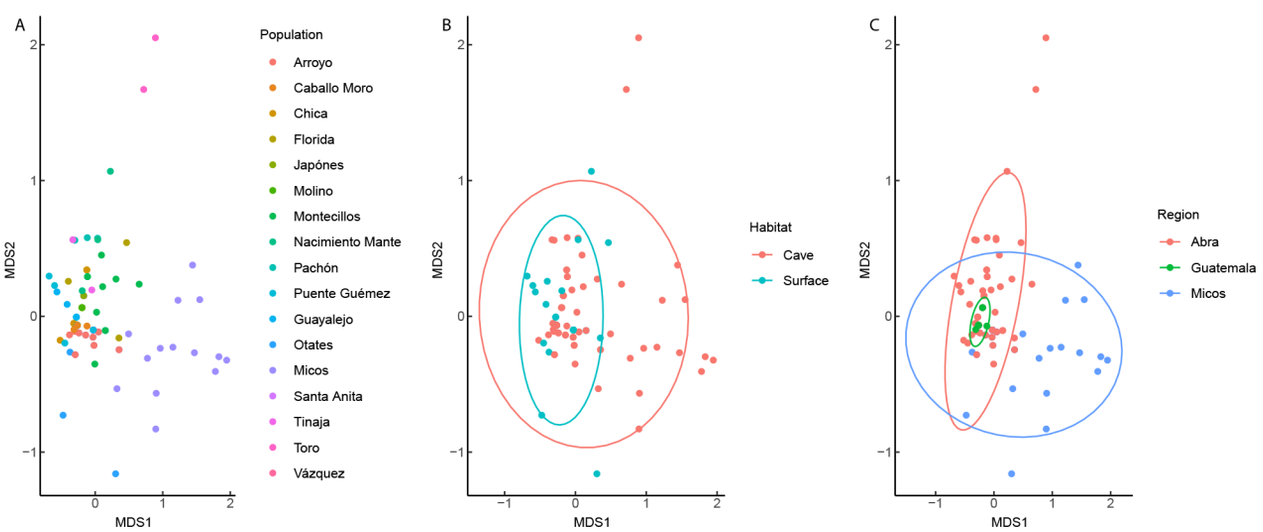


**Figure 5 Non-metric multidimensional scaling (NMDS) plot of 125 infracommunities based on Bray-Curtis distance**

A–C: Each point represents an infracommunity (all parasites within a host). Colors indicate host population (A); habitat (B); and geographical region (C). Stress: 0.100858.

host (see Herman et al., 2018). Another possibility is the presence of subterranean connections between cave populations (as suggested by Bradic et al., 2012), or that flooding events in some caves maintain gene flow between parasite populations. Additionally, the connectivity between surface and cave environments may contribute to the lack of differentiation between the surface and cave parasite lineages (see Moran et al., 2022). These phenomena are not mutually exclusive. However, it should be noted that our study was limited to a few genetic markers and a small number of parasite individuals. Conducting further analyses on other genomic regions would provide a more detailed picture of the evolutionary history of parasites and to test if they recover the same patterns and time frame of colonization as their hosts. Given their close association with cavefish and high prevalence, abundance, and intensity of infection, as well as their direct transmission route, monogeneans are prime candidates for investigating host-parasite phylogenetic congruence and coevolution. Both species of monogeneans found in the cavefish, i.e., *C*. cf. *chajuli* and *C*. cf. *costaricensis*, are considered species complexes, which will be described elsewhere (Santacruz, personal communication).

Research on parasites in troglobitic organisms has been limited, with only a few studies focusing on blind catfish (Moravec & Huffman, 1988) or cave animals such as guppies and salamanders (e.g., Dyer & Peck, 1975; Tobler et al., 2014). *Astyanax mexicanus* serves as a study model in the field of eco-evo-devo (Casane & Rétaux, 2016; Jeffery, 2020; Krishnan & Rohner, 2017). Thus, gaining an understanding of its ecological interactions in its natural environment is crucial for unraveling the mechanisms driving its adaptation to caves. The variation in parasite infections across different caves provides an invaluable opportunity to examine the role of parasites in the contrasting physiological, morphological, metabolic, and behavioral adaptive changes that have allowed *A. mexicanus* to colonize an extreme environment.

## CONCLUSIONS

We studied the parasites infecting the cavefish, providing the first parasitological records for more than half of their known populations. Our results indicate: (1) Great parasite diversity comprised by distantly related parasites, with contrasting life histories; (2) Most of the parasites found in the caves are a subset of the parasite diversity known for surface-dwelling populations; (3) Some parasite species are more frequent in the caves than in surface (i.e. monogeneans), making up the "cavefish core parasite fauna"; (4) The parasite communities vary across cavefish populations; (5) Infection patterns presented notable differences between cave and surface-dwelling populations. Additional caves remain to be explored to determine whether the patterns observed in our study persist. Nonetheless, our parasite inventory opens numerous avenues and questions regarding how host-parasite interactions are shaped in extreme cave environments. Further investigations that consider biotic variables in caves, together with the evolutionary history of parasites, should provide insights into the factors driving parasite diversification in caves.

## SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHOR'S CONTRIBUTIONS

A.S. conceived the study, contributed to fish sampling, performed the fish parasitological screening, identified the parasites, performed the experiments, analyzed the data, prepared the figures and/or tables, and wrote the paper. D.H.M. contributed to fish sampling and fish parasitological screening and reviewed and edited the manuscript. R.M.G. contributed to fish sampling. G.P.P.L. contributed to parasite identification, contributed reagents/materials, and reviewed and edited the manuscript. P.O.G. conceived the study, performed fish sampling, contributed reagents/materials, and reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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