Vol. 50: 17–30, 2023
https://doi.org/10.3354/esr01216

ENDANGERED SPECIES RESEARCH
Endang Species Res

Published January 19

# Estimating the population size and habitat quality of the Endangered fish *Tlaloc hildebrandi* in Mexico

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ABSTRACT: The Chiapas killifish *Tlaloc hildebrandi* is an Endangered and endemic fish that inhabits wetlands, mountain streams, and rivers in Chiapas, Mexico. This species is considered vulnerable due to accelerated human population growth in its distribution range and the species' low genetic diversity. To evaluate the conservation status of the species, we assessed habitat quality and estimated the population size of the remnant populations in the Amarillo River subbasin using the capture–mark–recapture technique. Our results showed substantial levels of habitat perturbation in the Amarillo River subbasin, including water pollution with a high presence of coliforms, the presence of exotic species, and modified habitat quality, which has resulted in a decrease in population sizes and the extirpation of certain populations. Our estimates of the population sizes of *T. hildebrandi* based on the Jolly-Seber model showed dramatically low population sizes, ranging from 93 to 208 fish across sites. Gross population sizes varied temporally, and the location of these populations in isolated sites may increase demographic stochasticity. To preserve some of these populations, urgent conservation and management activities must be implemented. We suggest the establishment of conservation areas for the species in the Fogótico River (which has the best water quality and habitat conditions) and habitat restoration in the protected areas of La Kisst and María Eugenia Mountain Wetlands, where populations of *T. hildebrandi* could be reintroduced. Finally, we propose the implementation of *ex situ* conservation programs to maintain genetic diversity and prevent local extinctions of the most vulnerable populations.

KEY WORDS: Capture–mark–recapture · Jolly-Seber model · Profundulidae · Endemic species · Conservation

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## 1. INTRODUCTION

Freshwater fish species are highly diverse, with over 18 000 species recognized worldwide (Fricke et al. 2021). However, this diversity is threatened by human activities, such as the construction of reservoirs (dams), habitat degradation and loss, the introduction of exotic species, overfishing, water pollution, and global climate change (Allan & Flecker 1993, Dudgeon et al. 2006, Dudgeon 2019). At present, it is estimated that at least one-third of the world's freshwater fishes are close to extinction, 80 species are extinct, and 10 species no longer exist in nature (Hughes 2021).

In Mexico, the freshwater ichthyofauna is comprised of 48 families and more than 600 species (Miller 2009, Contreras-MacBeath et al. 2014). Moreover, approximately 264 endemic species inhabit various basins throughout Mexican territory (Contreras-MacBeath et al. 2014). However, during the

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past 60 yr, there has been a decline in the numbers of some populations and species and the elimination of others, as well as an increase in the number of species at risk (Contreras-Balderas et al. 2008). Currently, there are 169 freshwater fish species in Mexico at some category of risk, of which 13 species are Extinct and 8 are Extinct in the Wild (IUCN 2021). Threatened species represent one-third of the country's ichthyofauna and are mainly endemic species with restricted distributions (Contreras-MacBeath et al. 2014).

Given the rapid deterioration of natural ecosystems, it is important to monitor endangered species and evaluate their biological attributes, such as population size and population trends over time (e.g. survival, recruitment, sex ratio, and size structure). It is also important to evaluate habitat availability and quality (Poos et al. 2012, Stewart et al. 2017, Hafen et al. 2021). This information facilitates estimation of population viability, identification of threats, evaluation of conservation status of species, and implementation of conservation and management actions to recover species through academic, social, and governmental sectors (Scheele et al. 2018, Lindenmayer et al. 2020).

*Tlaloc hildebrandi* (Cyprinodontiformes: Profundulidae) is an example of one of these endemic and threatened freshwater species. It is endemic to Mexico, where it is known as 'popoyote', 'escamudo de San Cristobal', or the Chiapas killifish. It is categorized as Endangered on the IUCN Red List (Schmitter-Soto & Vega-Cendejas 2019), by the American Fisheries Society (Jelks et al. 2008), and on the Official Federal List in Mexico (Norma Oficial Mexicana, NOM 059) (SEMARNAT 2019). This small killifish has been described as the only native species of Maria Eugenia Lake, a mountain wetland located in the limestone plateau at San Cristobal de Las Casas, Chiapas, that is now dry (Fig. 1) (Miller 1950, 1955). Until 2015, its distribution was limited to the wetlands, mountain streams, and rivers of the endorheic Amarillo River subbasin at Grijalva Basin (Velázquez-Velázquez & Schmitter-Soto 2004, Miller 2009). However, recent research has revealed *T. hildebrandi* in other locations, mainly in mountain streams and rivers in the Usumacinta and Grijalva Basins (Gómez-González et al. 2015, Velázquez-Velázquez et al. 2016, Domínguez-Cisneros et al. 2017, Beltrán-López et al. 2021, Calixto-Rojas et al. 2021). Nonetheless, all populations are geographically isolated, and the distribution of the species is restricted to an estimated area of 116 km$^2$ (Schmitter-Soto & Vega-Cendejas 2019).

The Chiapas killifish is one of the oldest members of the Profundulidae family, originating in the Lower Miocene due to geological instability in southern Mexico and Central America (Miller 1955, Doadrio et al. 1999, Morcillo et al. 2016). It is a species of small size that rarely exceeds a standard length (SL) of 12 cm. It has brown to dark coloration on the back and sides of the body, shades of yellow on the dorsal, anal, and caudal fins, and iridescent spots on the sides of the head (Miller 1950). It is an omnivorous species but largely consumes invertebrates such as insect larvae, crustaceans, amphipods, and mollusks. It reproduces mainly during the dry season (March to May) and exhibits sexual dimorphism. This species typically inhabits lentic and lotic areas with adequate oxygenation (mean dissolved oxygen 5.95 mg l$^{-1}$), although it is also found in shallow water that has a soft bottom. The Chiapas killifish is generally associated with rocky areas and vegetation that provides shelter and food (Velázquez-Velázquez et al. 2007).

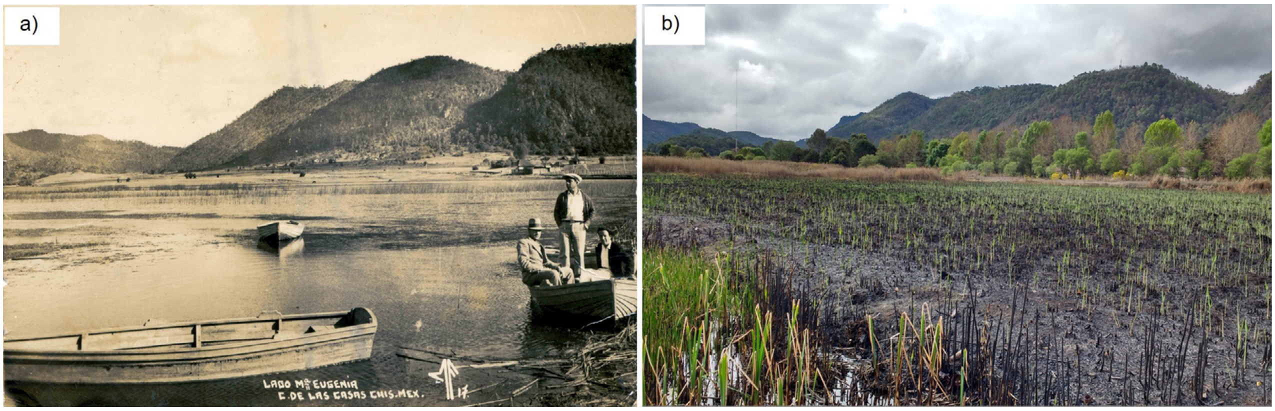

Fig. 1. María Eugenia Lake, the type locality of *Tlaloc hildebrandi*, (a) around 1940 and (b) at the location of the former lake in 2017. © José Antonio Crocker (~1940) and Alfonso A. González-Díaz

In 2000, population sizes of the Chiapas killifish in the Amarillo River subbasin were determined using the Lincoln-Petersen method. This method is based on a single capture and tagging event, followed by a subsequent recapture event of the marked animal, and it assumes that the population experiences no change in size during the sampling period (C. Krebs Ecological methodology, unpubl 3rd edn, https://www.zoology.ubc.ca/~krebs/books.html). Estimates indicated an extrapolated average population size of 40 000 individuals (95% confidence interval: 7745–72 047) (Velázquez-Velázquez & Schmitter-Soto 2004). The vulnerability of the species has been associated with factors resulting from urban growth, such as habitat loss and degradation, the contamination of water bodies, and the presence of exotic species and associated parasites (Velázquez-Velázquez & Schmitter-Soto 2004, Velázquez-Velázquez et al. 2011). Conservation efforts have included the establishment of 2 protected areas by the local government (SEMAVIHN 2010, 2011), La Kisst and María Eugenia Mountain Wetlands, which were recently declared critical habitats for the conservation of flora and fauna by the Mexican government (DOF 2022). Both sites have also been declared Ramsar sites of international importance, mainly because they are among the few mountain wetlands and are important habitats for waterbirds (SEMAVIHN 2010, 2011). Awareness-raising events highlighting the protection of the species and its habitat have also been carried out by local, academic, and governmental groups (Rodiles-Hernández et al. 2015). Previous studies have highlighted the need for information on the environmental requirements of the species and its populations in order to facilitate an understanding of historical and actual distributions in the region of the Chiapas highlands (Calixto-Rojas et al. 2021).

In recent years, there has been an increase in the human population of the distribution area of *T. hildebrandi.* The number of inhabitants in San Cristóbal de Las Casas increased from 99 254 in 1995 (Velázquez-Velázquez & Schmitter-Soto 2004) to 215 874 in 2020 (INEGI 2020). This situation has resulted in the growth of the urban area and the subsequent fragmentation, elimination, and pollution of the habitats of *T. hildebrandi*, causing a decrease in population sizes and an increase in the risk of extinction of the species (Rodiles-Hernández et al. 2015, Schmitter-Soto & Vega-Cendejas 2019). Recent genetic studies conducted on *T. hildebrandi* have revealed the critically low genetic diversity of the species, placing it among the most endangered species in Mexico. This genetic characterization identified 3 evolutionary significant units, one of which occurs in the Amarillo subbasin (Beltrán-López et al. 2021). The results of that study revealed the Amarillo River subbasin to be the least genetically diverse region.

Previous estimates of population size have been based on the data generated from the capture–mark–recapture technique using the Lincoln-Petersen method (Velázquez-Velázquez & Schmitter-Soto 2004), which is performed over short periods and considers the population geographically and demographically closed (C. Krebs Ecological methodology, unpubl 3rd edn, https://www.zoology.ubc.ca/~krebs/books.html). In contrast, by performing catches over a longer period, it is possible to detect population dynamics and gain a more accurate estimate of the population (Tazunoki et al. 2021). Data obtained through multiple captures are analyzed with models for open populations, such as the Jolly-Seber model, which considers the population to be affected by mortality, natality, and migration, and allows estimation of abundance, survival, recruitment, and probability of recapture (Schwarz & Arnason 2015). In this sense, the objective of the present work was to obtain an understanding of the conservation status of *T. hildebrandi* in the Amarillo River subbasin by estimating the population size through the capture–mark–recapture technique using the Jolly-Seber model and evaluating the quality of the habitat. Based on our results, we present several conservation proposals, including the establishment of potential areas for future reintroductions of the species and the selection of sites with improved environmental conditions for conservation and/or restoration in the medium term (8–10 yr).

## 2. MATERIALS AND METHODS

### 2.1. Study area

The distribution area of *Tlaloc hildebrandi* includes the mountain rivers and streams in the Altiplano Central or Altos de Chiapas Physiographic Province, Mexico (Miller 1955, Villalobos-Sánchez 2013). The species is distributed in the upper part of the Usumacinta and Grijalva basins, in the Tzaconejá and Jataté subbasins of the Usumacinta, and Chenalhó and Amarillo subbasins of the Grijalva (Domínguez-Cisneros et al. 2017, Schmitter-Soto & Vega-Cendejas 2019). The Amarillo River is an endorheic subbasin which has an area of 287.42 km$^2$ (Espíritu-Tlatempa & Rodiles-Hernández 2013). This subbasin is part of the municipality of San Cristóbal de Las Casas and includes part of the municipalities of Chamula, Huixtán, Tenejapa, and Zinacantán. It is in pine–oak and mesophyllous mountain forests. Hydrologically, it is composed of rivers, streams, and mountain wetlands (García 2015). Most of these water bodies such as the Amarillo, Fogótico, and Chamula Rivers and the Navajuelos and Ojo de Agua streams among others were historically inhabited by the Chiapas killifish (Fig. 2) (Velázquez-Velázquez & Schmitter-Soto 2004).

## 2.2. Environmental data

In February 2018, the sites in the Amarillo River subbasin previously reported for the Chiapas killifish (Velázquez-Velázquez & Schmitter-Soto 2004) were visited. However, specimens of *T. hildebrandi* were only observed at 4 sites: the Amarillo River (16° 44′ 39.02″ N, 92° 36′ 15.87″ W), Fogótico River (16° 44′ 3″ N, 92° 36′ 47.8″ W), Navajuelos stream at Parque de Los Humedales (16° 42′ 39.04″ N, 92° 37′ 32.51″ W), and Ojo de Agua stream (16° 45′ 11.38″ N, 92° 37′ 52.07″ W). These locations were used as sampling sites in the present study. The Chiapas killifish was not found at the remaining sites; anoxic conditions, extreme pollution, and exotic species were recorded (Table 1, Fig. 2).

Sampling was conducted from February to September 2018. During each month, 8 physicochemical variables were measured *in situ* at each sampling site. These included: water temperature (°C), pH, conductivity (mS $\mathrm{cm}^{-1}$), and total dissolved solids (mg $\mathrm{l}^{-1}$), which were recorded using a Hanna Instruments multiparameter meter (model HI9829). The dissolved oxygen concentration (mg $\mathrm{l}^{-1}$) and percentage saturation (%) was measured using a YSI 85 oximeter. These variables were recorded once at each sampling site during each month. The depth (cm) and current velocity (m $\mathrm{s}^{-1}$) were measured using a Global water FP101 flowmeter, and the transparency (cm) was measured using a Secchi disk. These 3 variables were recorded at 3 points within the sampling site, and an average value was obtained for each site during each month.

The physical habitat quality was assessed monthly at each site (February–September) using a rapid as-

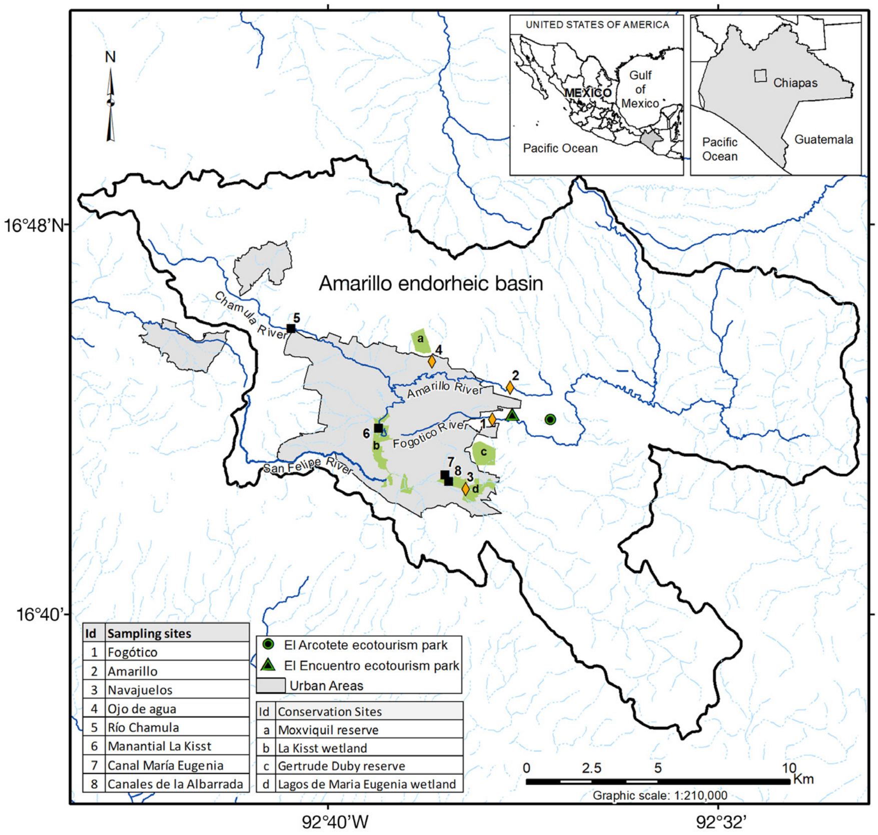

Fig. 2. Location of *Tlaloc hildebrandi* sampling sites in the Amarillo River subbasin. Sites 1–4 (orange diamonds) were visited monthly as part of this study, and sites 5–9 (black squares) were locations where the species was not found

Table 1. Environmental characteristics of sites visited in the Amarillo River subbasin where *Tlaloc hildebrandi* historically occurred and is now absent. T: temperature; TDS: total dissolved solids; DO: dissolved oxygen; MPN: most probable number

| Site | Coordinates | T (°C) | pH | Conductivity (μS cm$^{-1}$) | TDS (mg l$^{-1}$) | DO (mg l$^{-1}$) | Depth (cm) | Current velocity (m s$^{-1}$) | Observations |
|---|---|---|---|---|---|---|---|---|---|
| Chamula River | 16° 44′ 39.02″ N, 92° 36′ 15.87″ W | 17.62 | 7.94 | 647 | 490 | 5.63 | 18 | 22.8 | Coliforms $<7\,800\,000$ MPN per 100 ml |
| La Kisst Channel | 16° 45′ 52.16″ N, 92° 40′ 45.04″ W | 16.73 | 7.42 | 730 | 564 | 1.33 | 64 | 0 | *Micropterus salmoides*[a] |
| María Eugenia Channel | 16° 42′ 52″ N, 92° 37′ 35.76″ W | 10.53 | 8.52 | 859 | 773 | 3.24 | 20 | 0 | *Poeciliopsis hnilickai*[a] |
| La Albarrada Channels | 16° 42′ 44.39″ N, 92° 37′ 31.47″ W | 13.2 | 8.08 | 505 | 252 | 6.4 | 44 | 0.45 | *Cyprinus carpio*[a], *Procambarus clarkii*[a] |

[a]Exotic species caught

sessment protocol (Barbour et al. 1991). This assessment is used in wadable rivers and streams and is based on the analysis of physical characteristics of the water body and the riparian areas in which it is located, as these parameters are related to the development of aquatic life. At each sampling site, a 100 m transect was established along the stream or river. Ten parameters were evaluated: the available cover, substrate embeddedness, velocity/depth regimes, sediment deposition, channel flow status, channel alteration, frequency of riffles, bank stability, vegetative protection, and riparian vegetative zone width (Table S1 in the Supplement at www.int-res.com/articles/suppl/n050p017_supp.pdf). Each parameter was evaluated on a numerical scale from 0 to 20, which indicated a condition category: optimal (20–16), suboptimal (15–11), marginal (10–6), or poor (5–F0); the total score is obtained by adding up the scores of all parameters. To validate the procedure, 3 people performed the assessment at each site per month, following the description and criteria provided by the protocol, and then the scores were averaged by month.

Water samples were collected at each site in February, April, June, and September in order to determine the water chemistry. These samples were transported to the ECOSUR Institutional Laboratory (Chiapas, Mexico) for processing. Total hardness (mg $CaCO_3$ l$^{-1}$) was determined by the volumetric method (Baird & Eaton 2017). Alkalinity (mg $CaCO_3$ l$^{-1}$) was determined according to the method of NMX-AA-036-SCFI-2001 (2001). Turbidity (NTU) was measured using a radiation attenuation method. Suspended solids (SS, mg l$^{-1}$) were determined by the photometric method (Baird & Eaton 2017). Nitrates (mg l$^{-1}$) were determined by the cadmium reduction method and nitrites (mg l$^{-1}$) by diazotization (Baird & Eaton 2017). Ammonium concentration (mg l$^{-1}$) was measured using the salicylate method (Reardon et al. 1966). Reactive phosphorus as phosphates (mg l$^{-1}$) was determined by the salicylic acid method (Baird & Eaton 2017). Chemical oxygen demand (mg $O_2$ l$^{-1}$) by reflux digestion was carried out using a procedure adapted from the Environmental Protection Agency (EPA) Method 410.4. Biochemical oxygen demand ($BOD_5$, mg $O_2$ l$^{-1}$) was calculated using a respirometric method (Baird & Eaton 2017). Total coliforms (most probable number, MPN 100 ml$^{-1}$), and fecal coliforms (fecal *Escherichia coli*, MPN 100 ml$^{-1}$) were determined with an enzyme-defined substrate test (Baird & Eaton 2017).

### 2.3. Fish sampling

The sampling of fish was conducted monthly from February to September. The area sampled at all sites consisted of a 100 m transect. Sampling was conducted in the morning for 3 or 4 h. The areas sampled included the shorelines of rivers and streams, as well as other available habitat types: pools, riffles, and runs with cover provided by rocks or vegetation. The specimens were captured along the 100 m transect using ETS backpack electrofishing (ETS Electrofishing Model ABP-3TM) with a 150 V discharge. The captured fish were anesthetized with clove oil drops for ease of handling. Subsequently, the sex of each specimen was determined. Males have a longer anal fin base, and the shape of the anal fin is more rounded, i.e. the anterior rays of the anal fin are not

Fig. 3. Chiapas killifish with a fluorescent visible implant elastomer (VIE) in the caudal peduncle (red)

as long as the posterior ones (Miller 1950). Specimens that could not be differentiated by fin characteristics and with standard length (SL) <5 cm were considered juveniles. Each specimen was photographed (using a Sony camera, model SLT-A37), a scale was placed on each photograph, and the SL was obtained using tpsDig2 ver. 2.32 software (Rohlf 2021).

The population size was estimated for each site using the capture–mark–recapture technique. Each fish that was caught was tagged using a fluorescent visible implant elastomer (VIE; North Marine Technology). Before sampling, the body location of the VIE and the color were selected in the laboratory for improved visualization (Fig. 3). Four colors of VIE tags were used, with color and location of the tags being different for each of the 8 mo. The tags were injected at the base of the anal fin (February–May) and at the base of the caudal fin (June–September). A different tag color was used for each month in the following order: red, pink, yellow, and orange; the same color order was then repeated.

To meet the assumptions of the capture–mark–recapture method in open population models, individuals were marked with specific tags at each sampling time (C. Krebs Ecological methodology, unpubl 3rd edn, https://www.zoology.ubc.ca/~krebs/books.html), in this case, tags differed by VIE coloration and location. The tagged fish were returned to the environment at the same point of capture. During sampling, no specimens were killed by the collection method (electrofishing) or by manipulation during tagging. The tracking of individual tags was based on the sex, size, coloration, and location of the VIE during recapture.

### 2.4. Data analysis

The physicochemical variables and habitat quality scores obtained monthly for each site were log-transformed (log [$x$ + 1]) and analyzed via principal component analysis (PCA), using a correlation matrix, to recover an ordination of the sampling sites according to those sets of variables and to explore temporal variation. These analyses were performed using R version 3.6.3 (R Core Team 2021) and the 'vegan' package version 2.5-7 (Oksanen et al. 2019). Regarding the interpretation of the results of the PCA, variables with loadings greater than 0.6 were considered significant (Sedeño-Díaz & López-López 2007).

The water chemistry variables, obtained on only 4 occasions, were compared between sites. These parameters were log-transformed (log [$x$ + 1]) because they did not present a normal distribution. The comparison was made using a non-parametric Kruskal-Wallis (KW) test, and pairwise comparisons were made using the Wilcoxon rank-sum test. These analyses were performed using R version 3.6.3 (R Core Team 2021).

The capture–mark–recapture data were analyzed using an open population model, which assumes that the population size is not constant. The 'POPAN' formulation of the Jolly-Seber model was used in MARK software version 9.0. This formulation was selected because it can estimate the population size at each sampling time per site. It calculates the probability of capture (p) of tagged and untagged organisms, the probability of apparent survival (φ), and the probability of entering the population (β, pent), from which it estimates the population size (N). This model generates a 95% confidence interval for each population size estimate (Schwarz & Arnason 2015). If every fish marked in the sample is recaptured, the probability of capture (p-value) is 1. It also assumes that all individuals have the same probability of capture. Survival takes a value of 1 if there is no mortality or emigration (Pine et al. 2003).

For each site, 4 models were generated considering that p, φ, and β varied over time; p was constant; φ was constant; and p and φ were constant. A model was selected based on the corrected Akaike's information criterion ($AIC_C$), for which a lower value indicates a better fit (Schwarz & Arnason 2015). From the selected model, we obtained an estimate of the population size at each site.

## 3. RESULTS

### 3.1. Environmental analysis

The PCA showed segregation between the sites related to physicochemical variables and habitat quality (Fig. 4). The first 2 axes of the PCA explained 65.38% of the variation (Table S2). PC1 separated the Ojo de Agua and Navajuelos streams due to higher conductivity values and higher concentrations of total dissolved solids. In contrast, the Amarillo and Fogótico Rivers were characterized by higher pH values, higher dissolved oxygen concentrations, and higher habitat quality (Fig. 5, Table 2). PC2 separated the Ojo de Agua stream towards the negative end of the axis, which was associated with greater transparency and shallower depth (Table 2).

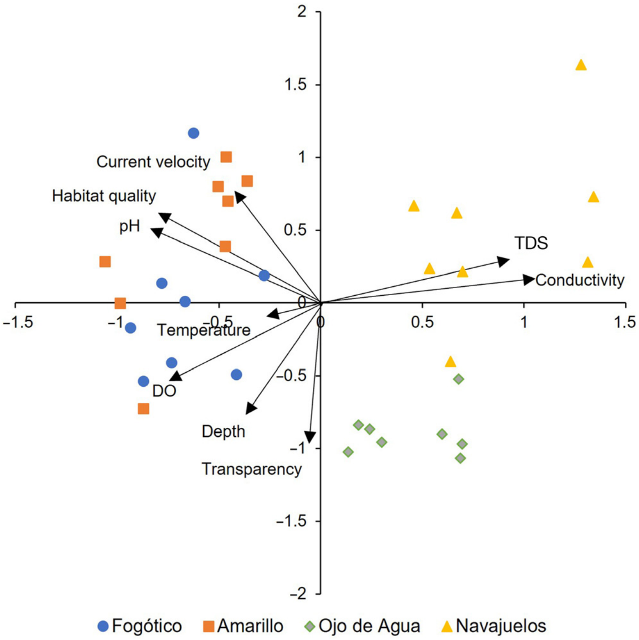

Fig. 4. Principal component analysis ordination showing the 2 first components of variation among sampling sites in the Amarillo River subbasin. Each point corresponds to 1 month of sampling. TDS: total dissolved solids

The results of the water quality analysis indicated significant differences in hardness among the study sites (KW = 12.706, p = 0.005). The highest values were recorded in the Navajuelos and Ojo de Agua streams (301.3 and 174.7 mg $l^{-1}$, respectively, p = 0.03). Similarly, significant differences in alkalinity were observed between the sites (KW = 12.728, p = 0.005). The highest concentrations were recorded in the Navajuelos and Ojo de Agua streams (303.1 and 187.6 mg $l^{-1}$, respectively, p = 0.03). Additionally, significant differences were found in nitrate concentrations between the locations (KW = 9.7257, p = 0.02). The highest value was recorded at the Ojo de Agua stream (0.91 mg $l^{-1}$), which was significantly different from the Fogótico River (p = 0.03) and the Navajuelos stream (p = 0.03). The remaining parameters did not vary significantly between sites. There were low concentrations of nitrites (average value of 0.25 mg $l^{-1}$), ammonium (0.01–0.03 mg $l^{-1}$), and phosphates (0.11–0.06 mg $l^{-1}$), and, notably, coliforms were detected at all sites (295–1490 MPN per 100 ml) (Table 3).

### 3.2. Population size

*Tlaloc hildebrandi* was the only fish species present in most of the study areas, except for the Ojo de Agua Stream, where 2 specimens of the Characidae

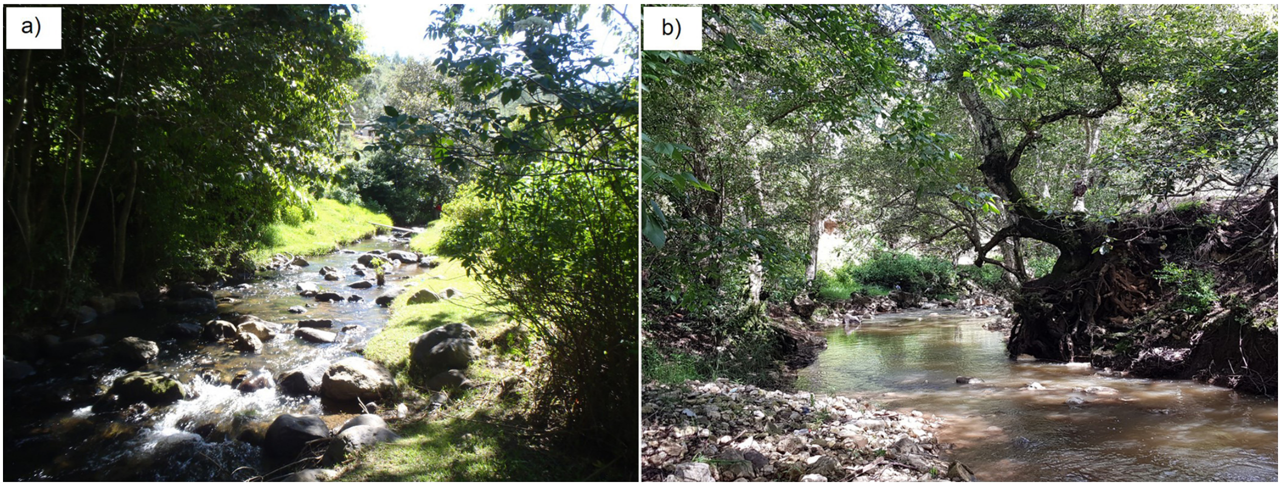

Fig. 5. Sampling sites with high habitat quality scores: (a) Amarillo River and (b) Fogótico River

Table 2. Physicochemical variables and habitat quality of sampling sites of *Tlaloc hildebrandi* observed during the study period. Values are means (SD). TDS: total dissolved solids

| | Fogótico River | Amarillo River | Ojo de Agua stream | Navajuelos stream |
|---|---|---|---|---|
| Temperature (°C) | 15.9 (0.9) | 14.7 (1.1) | 15.2 (0.2) | 13.3 (2.2) |
| pH | 8.4 (0.2) | 8.3 (0.2) | 7.5 (0.2) | 7.8 (0.1) |
| Conductivity (µS $cm^{-1}$) | 217 (62) | 214 (57) | 404 (96) | 602 (155) |
| TDS (mg $l^{-1}$) | 149 (75) | 146 (69) | 275 (125) | 394 (205) |
| Dissolved oxygen (mg $l^{-1}$) | 7.8 (0.3) | 8.0 (0.4) | 8.2 (0.5) | 5.5 (0.5) |
| Depth (cm) | 38 (7) | 33 (10) | 38 (4) | 27 (7) |
| Current velocity (cm $s^{-1}$) | 8.8 (7.4) | 21.3 (9.4) | 5.5 (1.8) | 8.1 (4.6) |
| Transparency (cm) | 32 (11) | 30 (11) | 38 (4) | 27 (7) |
| Habitat quality | 134 (8) | 120 (6) | 30 (1) | 61 (2) |

Table 3. Water chemistry for the sampling sites of *Tlaloc hildebrandi*. Values are means (SD) of February, April, June, and September. Asterisks indicate significant ($p < 0.05$) differences. COD: chemical oxygen demand; $BOD_5$: biochemical oxygen demand; MPN: most probable number

| | Fogótico River | Amarillo River | Ojo de Agua stream | Navajuelos stream |
|---|---|---|---|---|
| Hardness (mg $CaCO_3$ $l^{-1}$) | 101.7 (17.3) | 97.5 (15.3) | 174.7 (31.6)* | 301.3 (37.8)* |
| Alkalinity (mg $CaCo_3$ $l^{-1}$) | 91.8 (9.9) | 97.2 (19.8) | 187.6 (17.7)* | 303.1 (18.6)* |
| Turbidity (NTU) | 8.6 (12.0) | 15.8 (16.3) | 7.1 (10.1) | 4 (2.0) |
| Suspended solids (mg $l^{-1}$) | 8.7 (10.0) | 10.8 (14.9) | 5.3 (5.3) | 3.9 (2.8) |
| Nitrates (mg $l^{-1}$) | 0.06 (0.04) | 0.14 (0.12) | 0.91 (0.70)* | 0.05 (0.03) |
| Nitrites (mg $l^{-1}$) | 0.25 (0.50) | 0.25 (0.50) | 0.25 (0.50) | 0.25 (0.50) |
| Ammonium (mg $l^{-1}$) | 0.02 (0.02) | 0.01 (0.01) | 0.03 (0.03) | 0.03 (0.03) |
| Phosphates (mg $l^{-1}$) | 0.11 (0.09) | 0.08 (0.04) | 0.07 (0.01) | 0.06 (0.06) |
| COD (mg $O_2$ $l^{-1}$) | 11.8 (10.3) | 9 (4.7) | 3.5 (2.1) | 4.1 (3.1) |
| $BOD_5$ (mg $O_2$ $l^{-1}$) | 4.9 (5.9) | 2.8 (1.9) | 1.3 (1.7) | 1.4 (1.9) |
| Total coliforms (MPN 100 $ml^{-1}$) | 295 (247.3) | 1040 (1049.7) | 1490 (2052.7) | 830 (671.4) |
| Fecal coliforms (MPN 100 $ml^{-1}$) | 50 (25.8) | 235 (157.8) | 1310 (1996.6) | 575 (779.0) |

Table 4. Specimens of *Tlaloc hildebrandi* caught from February to September 2018 by sampling sites. The number of individuals released was the same as the number captured. Standard length (SL) with minimum and maximum values

| Site | Specimens | February | March | April | May | June | July | August | September | Total | SL (mm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fogótico River | Caught | 20 | 26 | 12 | 9 | 2 | 5 | 13 | 7 | 94 | 21–103 |
| | Recaptured | 0 | 4 | 8 | 3 | 0 | 2 | 7 | 1 | 25 | |
| | Marked | 20 | 22 | 4 | 6 | 2 | 3 | 6 | 6 | 69 | |
| Amarillo River | Caught | 22 | 12 | 17 | 11 | 1 | 15 | 12 | 11 | 101 | 26–111 |
| | Recaptured | 0 | 3 | 2 | 6 | 0 | 3 | 7 | 4 | 25 | |
| | Marked | 22 | 9 | 15 | 5 | 1 | 12 | 5 | 7 | 76 | |
| Ojo de Agua stream | Caught | 43 | 31 | 21 | 29 | 24 | 13 | 15 | 12 | 188 | 35–119 |
| | Recaptured | 0 | 5 | 6 | 14 | 16 | 4 | 12 | 10 | 67 | |
| | Marked | 43 | 26 | 15 | 15 | 8 | 9 | 3 | 2 | 121 | |
| Navajuelos stream | Caught | 9 | 4 | 6 | 9 | 5 | 1 | 1 | 0 | 35 | 24–110 |
| | Recaptured | 0 | 0 | 1 | 0 | 4 | 1 | 1 | 0 | 7 | |
| | Marked | 9 | 4 | 5 | 9 | 1 | 0 | 0 | 0 | 28 | |

family (cf. *Astyanax*) were collected, and the Navajuelos Stream, where 7 specimens of the common carp *Cyprinus carpio* were collected. A total of 420 Chiapas killifish were collected at the 4 sampling sites during an 8 mo period; however, no fish were collected at the Navajuelos stream in September (Table 4). The Ojo de Agua stream had the highest number of fish caught and recaptured (188 and 67 fish, respectively), and the Navajuelos stream had the lowest number (35 and 7 fish, respectively). The lengths of the captured fish were similar between sites and ranged from 21 to 119 mm SL.

In the Amarillo River, Ojo de Agua and Navajuelos streams, the best fitted POPAN model was p(.)*phi(.)*pent(t)*N(t), which contained constant survival (φ) and capture probability (p) over time (marked as a dot), and included the probability of entry (pent) and super-population size over time. For the Fogótico River, this model was the second best fit, but we considered this model to be the most appropriate for the data (Table S3). At all sites, the estimated survival rates were high and capture probability was low. For the Fogótico River, $\varphi = 91\%$ and $p = 21\%$; for the Amarillo River, $\varphi = 94\%$ and $p = 17\%$; for the Ojo de Agua Stream, $\varphi = 96\%$ and $p = 18\%$; and for the Navajuelos Stream, $\varphi = 93\%$ and $p = 12\%$ (Table S4).

The estimated population size fluctuated over the time of sampling (Table 5). The highest monthly estimates were observed for the Ojo de Agua stream, ranging from N = 208 in February to 74 in September. The Amarillo River varied from N = 110 to 58. For the Fogótico River, the monthly estimate varied from N = 94 to 40, and the lowest estimates were observed for the Navajuelos stream, which ranged from N = 63 to 21.

Overall, the population size gross estimates indicated that the Ojo de Agua stream was the site that had the largest population size, with an average estimate of 208 fish (95% CI: 173–250), followed by the Amarillo River with 191 fish (95% CI: 135–271) and the Fogótico River with 187 fish (95% CI: 131–267). The smallest population size was estimated for the Navajuelos stream, at 93 fish (95% CI: 49–178) (Table 5).

Table 5. Population size estimates of *Tlaloc hildebrandi* obtained from the Jolly-Seber models (POPAN in MARK v. 9.0) for each sampling site. Values by month and gross population, with 95% confidence intervals

| Site | Month | Population estimate | Upper limit | Lower limit |
|---|---|---|---|---|
| Fogótico River | February | 94 | 170 | 52 |
| | March | 99 | 144 | 69 |
| | April | 67 | 102 | 44 |
| | May | 45 | 76 | 27 |
| | June | 30 | 57 | 16 |
| | July | 28 | 56 | 14 |
| | August | 39 | 79 | 20 |
| | September | 40 | 78 | 20 |
| | Gross population | 187 | 267 | 131 |
| Amarillo River | February | 110 | 185 | 66 |
| | March | 85 | 153 | 47 |
| | April | 80 | 122 | 53 |
| | May | 62 | 102 | 37 |
| | June | 47 | 87 | 26 |
| | July | 81 | 135 | 49 |
| | August | 62 | 114 | 34 |
| | September | 58 | 111 | 30 |
| | Gross population | 191 | 271 | 135 |
| Ojo de Agua stream | February | 208 | 250 | 173 |
| | March | 179 | 212 | 151 |
| | April | 155 | 183 | 130 |
| | May | 133 | 161 | 110 |
| | June | 115 | 143 | 92 |
| | July | 99 | 128 | 76 |
| | August | 85 | 116 | 63 |
| | September | 74 | 105 | 52 |
| | Gross population | 208 | 250 | 173 |
| Navajuelos stream | February | 63 | 151 | 26 |
| | March | 47 | 130 | 17 |
| | April | 48 | 130 | 18 |
| | May | 50 | 98 | 26 |
| | June | 38 | 85 | 17 |
| | July | 28 | 77 | 11 |
| | August | 21 | 71 | 7 |
| | Gross population | 93 | 178 | 49 |

## 4. DISCUSSION

The Chiapas killifish holds a critical conservation status in the Amarillo River subbasin. This is due to changes in land use, population growth, and rapid urbanization. Our results indicated that some of the Chiapas killifish populations in the Amarillo River subbasin have been eliminated due to the presence of contamination and exotic species. In this study, all sampling sites showed coliform contamination; the Fogótico and Amarillo Rivers showed similar physicochemical conditions and a higher habitat quality score compared to Ojo de Agua and Navajuelos streams. However, the estimated population size indicates the presence of few individuals at all sites, with site estimates ranging from 93 to 208 fish. All of these populations are isolated. Several of the major threats to the conservation of the species are described below.

The physicochemical variables of the water and habitat quality score differed among the 4 study sites. The Fogótico and Amarillo Rivers presented optimal conditions for the conservation of the species with regard to dissolved oxygen and pH, as well as the presence of habitat heterogeneity (i.e. pools, rapids, rocky areas, and riparian vegetation). Together, these factors could offer the ideal habitat conditions for the establishment and protection of the Chiapas killifish (Velázquez-Velázquez & Schmitter-Soto 2004). The Fogótico and Amarillo Rivers are the main tributaries in the subbasin (García 2015); however, the Amarillo River is the narrowest and is characterized by higher current speeds due to altitudinal changes (approximately 770 m) (Espíritu-Tlatempa & Rodiles-Hernández 2013). However, during the sampling period this tributary showed a decrease in riparian vegetation and an increase in the number of human settlements along its course. The Fogótico River is in an area that has a lower altitude difference and has a wider channel and greater coverage of riparian vegetation (mainly natural patches of pine–oak forest). This river crosses 2 natural ecotourism parks, the 'El Arcotete' (communal property) and 'El Encuentro' (small private reserve), which favor habitat conservation (Fig. 2).

In contrast, the Ojo de Agua and Navajuelos streams showed poorer habitat quality. Their natural structure has been modified by canalization and water extraction; the predominant type of substrate was mud, the water level was generally low, and the natural riparian vegetation coverage was low. Both streams showed high levels of hardness, alkalinity, conductivity, and total dissolved solids. These physicochemical variables are associated with the geomorphological nature of the basin, which is composed of limestone rocks (García 2015). However, the increased concentrations may be due to human activities (Castillo et al. 2012), as both streams are located within urban areas.

Water chemistry analyses showed high nitrogen concentrations. For example, high concentrations of nitrates were recorded in the Ojo de Agua stream, and high levels of nitrites were recorded at the other sites. This is associated with the presence of surrounding agricultural areas. Moreover, the concentrations of these nitrogen forms may increase during the rainy season due to run-off and the incorporation of fertilizers into the aquatic ecosystems (Sedeño-Díaz & López-López 2007, Castillo et al. 2012). Furthermore, total and fecal coliforms were recorded at all sampled sites, which may be attributed to the incorporation of urban sewage inputs into the water bodies and agricultural activities in the subbasin (Castillo et al. 2012, García 2015, Camacho-Valdez et al. 2019).

Capture by electrofishing and tagging with VIE elastomer proved to be a reliable and non-invasive technique in this Chiapas killifish capture–mark–recapture study. Its application allowed assumptions for models in open populations to be fulfilled: the tags are permanent, and the use of colors and their location allowed differentiation and individual tracking of the recaptured organisms (C. Krebs Ecological methodology, unpubl 3rd edn, https://www.zoology.ubc.ca/~krebs/books.html). During the capture and handling of the organisms, none suffered any damage or mortality, so the tags did not change the probability of capture of the organisms or the probability of survival.

Demographic parameters are essential for monitoring endangered species; however, population model estimates are dependent on capture and recapture abundances (Schwarz & Arnason 2015). Despite the great effort applied during the collection of samples, only 420 fish were captured during the entire sampling period. This number is low compared to previous catches in the subbasin which ranged from 486 specimens collected in 1941 in the María Eugenia Lake (Miller 1955) to 1310 specimens collected in 2006 and 2007 (Velázquez-Velázquez et al. 2011), indicating a drastic reduction in the Chiapas killifish population size, which could put this species in danger due to demographic stochasticity (Lande 1988, Elgar & Clode 2001, Ornelas-García et al. 2012). The POPAN formulation of the Jolly-Seber models showed seasonal variation in the population size, a high probability of survival, a low probability of capture, and a low estimate of the gross population size. Unlike closed population models, this open model considers changes in the population (produced by recruitment, immigration, death, or emigration) and generates more accurate estimates of population size (Pine et al. 2003, Schwarz & Arnason 2015). However, the limitation of the model is that it assumes that the probability of capture is equal over time, which produces a bias in the population size estimates (Pollock 2002). In this study, during the rainy season, the sampling and capture were made more difficult by the increase in volume, depth, and speed of the current. These environmental seasonal changes influence the local abundance and habitat use of fish populations (Tazunoki et al. 2021).

The population sizes obtained for each site were lower compared with the estimates of the population size obtained 21 yr prior, which estimated a population size of 40 000 individuals in the Valley of San Cristóbal (Velázquez-Velázquez & Schmitter-Soto 2004). Moreover, an absence of Chiapas killifish in several locations in the subbasin was confirmed during the period of this study; this occurred, for example, in the Chamula stream, the Kisst Wetland, and the Albarrada and María Eugenia channels. Certain sites, such as the stream and channel in María Eugenia Lake (Velázquez-Velázquez & Schmitter-Soto 2004), suffered drastic physicochemical perturbations, which resulted in the loss of Chiapas killifish populations. The same conditions have been observed in the Chamula stream, which now transports wastewater. Additionally, we observed the presence of exotic species like largemouth bass *Micropterus salmoides*, red swamp crayfish *Procambarus clarkii*, Upper Grijalva livebearer *Poeciliopsis hnilickai*, and common carp.

The Fogótico and Amarillo Rivers showed similar population size estimates and available habitat for the species. However, during field trips, we detected an absence of the Chiapas killifish in several sections of the Fogótico River, which was likely due to the fragmentation of the river channel in the upper parts of the basin, and the presence of exotic species, such as rainbow trout *Oncorhynchus mykiss*, which is a predator of Chiapas killifish (pers. comm. from fishermen [names unknown]). The high current speeds ($>50$ m $s^{-1}$) in the Amarillo River do not provide an ideal habitat for the species. Also, the headwaters are more vulnerable to the effects of the changes in land use due to agriculture, deforestation, and the continuous establishment of urban settlements on the riverbank (Figueroa-Jáuregui et al. 2011, Castillo et al. 2012, Vásquez-Moreno & Córdova 2013, García 2015).

In accordance with its low habitat quality, the Navajuelos stream showed the lowest population size estimates. However, the Ojo de Agua stream had poor habitat and water quality, but the highest population size estimates, making this population very vulnerable to local extinctions due to habitat conditions. Additionally, a greater abundance of exotic fish was observed in these streams. For example, common carp were caught in the Navajuelos stream. This species is considered to be highly invasive and can have a negative impact on Chiapas killifish through the transmission of harmful parasites to juveniles (Velázquez-Velázquez et al. 2011). In the Ojo de Agua stream, fish from the family Characidae (cf. *Astyanax*) were caught. Members of this family have been introduced due to activities associated with the hobby of aquarium keeping. This species is a potential predator of cyprinodontid eggs (Ornelas-García et al. 2018).

The Chiapas killifish habitat is threatened and affected by population growth. Between 2001 and 2018, urban growth in the Amarillo River subbasin was recorded to be 12.5% (Camacho-Valdez et al. 2019). There is now a greater number of human settlements in the upper parts of the subbasin, and 7.5% of the wetlands have been lost due to anthropogenic activities (Camacho-Valdez et al. 2019). In the area of the rivers approaching the urban area, conditions for the survival of the Chiapas killifish are diminished due to lost habitat and water pollution, which is increased by the direct discharge of wastewater from the city of San Cristóbal de Las Casas and neighboring towns. Moreover, there are no water treatment plants, and the sewerage system is poorly designed and in disrepair (García 2015).

At present, the Chiapas killifish populations in the Amarillo River basin are isolated and fragmented. Lack of connectivity among the areas where specimens can still be observed could lead to the loss of genetic diversity and an increase in the vulnerability of the species to extinction (Beltrán-López et al. 2021). If the factors that affect the population size are maintained or the conditions worsen, this could promote demographic stochasticity of the species, increasing the risk of local extirpation in the Amarillo River subbasin, as has been the case for other endemic freshwater species in Mexico (Ornelas-García et al. 2012, Lyons et al. 2020, Valdés González et al. 2020).

Our results were limited to an 8 mo population assessment in the Amarillo River subbasin. To understand the population dynamics of *Tlaloc hildebrandi* throughout its range, it is necessary to assess populations in rivers and streams in the Tzaconejá, Jatatatá, and Chenalhó subbasins. In addition, an analysis of temporal changes spanning larger scales (years) is required (Stewart et al. 2017). We recommend the application of a combined capture–mark–recapture technique, which includes methods for closed and open populations (C. Krebs Ecological methodology, unpubl 3rd edn, https://www.zoology.ubc.ca/~krebs/books.html) and allows for obtaining a more robust estimate of population changes.

## 5. CONCLUSIONS

The Chiapas killifish has become more vulnerable to extinction due to habitat loss and interaction with exotic species. However, the species possesses some biological characteristics that may reduce its susceptibility to extinction processes (Olden et al. 2008, le Feuvre et al. 2021), such as an omnivorous diet (Velázquez-Velázquez et al. 2007), a tolerance for living in disturbed habitats as evidenced in this study, and the ability to reproduce at small sizes (around 6.5 cm SL; pers. obs.). The *ex situ* reproduction of Chiapas killifish and the subsequent reintroduction to conserved or restored sites is required, not only to preserve the threatened populations, but also to maintain genetic diversity. The Amarillo River subbasin represents a unique evolutionary significant unit that is crucial for the conservation of the species.

To support *in situ* conservation of Mexican freshwater fish, various academic and research institutions have initiated *ex situ* conservation strategies, involving the creation of living collections that ensure survival and reproduction in captivity (Lascuráin et al. 2009). This strategy seeks to recover the ecological and biological functioning of ecosystems, increase the size of natural populations (Conway 1989), maintain genetic variability, and avoid non-viable or undesirable genetic mixtures (Bernos et al. 2020). *Ex situ* conservation has been successful in other cases with Mexican fishes (Maceda-Veiga et al. 2016), including the killifishes *Profundulus oaxacae*, *P. mixtlanensis*, and *P. adani* (https://profundulusoaxacae.jimdofree.com/). Additionally, the success of *ex situ* conservation has allowed the reintroduction of other species, such as the Tequila splitfin *Zoogoneticus tequila* (Domínguez-Domínguez et al. 2018).

We recommend the establishment of protected areas along the Fogótico and Amarillo Rivers for the management and conservation of the species in the Amarillo River subbasin. These areas contain suitable habitats for the species, with a rocky substratum and adequate riparian and submerged vegetation, as well as moderate to fast water current speeds. Furthermore, the habitats of the protected natural areas, La Kisst and María Eugenia Mountain Wetlands, as well as the Ojo de Agua and Navajuelos streams must be restored. These ecosystems are part of the historical distribution of the species, and some of them could be used as refuge sites (i.e. 'ark' sites) (Nightingale et al. 2017) in the Amarillo River subbasin. International institutions suggest that conservation activities related to the propagation, translocation, reintroduction, and augmentation of imperiled fish should be implemented in their historical distribution range (George et al. 2009). Additionally, the regulation and reduction of anthropogenic activities in the subbasin would greatly contribute to the conservation of the species. It is particularly important to control the spread of introduced fish species by implementing actions to reduce the population sizes of these species in the Amarillo River subbasin. The eradication or control of populations of non-native species is a fundamental part of the success of freshwater fish conservation actions (George et al. 2009).

Finally, the success of proposals aimed at the conservation *in situ* and *ex situ* of Chiapas killifish and its habitat requires the participation of various sectors of academia, society, and government. For this, the creation of a network of governmental, non-governmental, public, and private institutions that coordinate the planning, design, and execution of conservation activities (as well as collaborate in decision-making) would be essential (George et al. 2009, Miqueleiz et al. 2020). This would include the implementation and dissemination of appropriate policies, strategies, and legislation (George et al. 2009, Reid et al. 2013).

*Acknowledgements*. The project was funded by The Mohamed bin Zayed Species Conservation Fund (Project 162513827). The support of the project 'Connectivity and functional diversity of the Usumacinta River basin' (FID-ECOSUR 784-1004) is gratefully acknowledged. The fish were caught under fishing permit SEMARNAT-SGA/DGVS/01283/20. We thank the anonymous reviewers for their helpful comments and observations to improve this manuscript; Allison A. Pease for the English review; Guadalupe García, Yanet Aguilar, and Melquiades Solís for their support in the field work; Juan Morales for his support in determining the water quality parameters; Julio Llanes for making the map; biologist Lemus Kurchenko for access to sampling sites of the municipal government; and the Decker family for access to their property at Ojo de Agua stream.

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*Editorial responsibility: Constance M. O'Connor, Toronto, Ontario, Canada,*
*Reviewed by: N. Mercado Silva, E. Contreras-MacBeath and 1 anonymous referee*

*Submitted: December 16, 2021*
*Accepted: November 17, 2022*
*Proofs received from author(s): December 24, 2022*