Biotecnia
Journal of biological and health sciences
http://biotecnia.unison.mx
Universidad de Sonora
ISSN: 1665-1456

Evidence of morphological variation in an isolated refuge population of the Sonoyta pupfish (*Cyprinodon eremus*) (Teleostei: Cyprinodontidae)

Evidencia de variación morfológica en una población de refugio aislada del cachorrito de Sonoyta (*Cyprinodon eremus*) (Teleostei: Cyprinodontidae)

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ABSTRACT

The Sonoyta pupfish (*Cyprinodon eremus*) is an endangered species endemic to the Sonoyta River basin, in northwestern México and southwestern United States. To assist the conservation efforts for this species in México, a refuge population was established in an artificial pond in 1988 at Centro Ecológico de Sonora in Hermosillo, Sonora by translocating individuals from the Sonoyta River population. We used multivariate morphometric methods to delineate body shape variations in the refuge population after 29 years of isolation, in comparison with wild individuals collected from the same sample. Significant variations were observed in body shape between refuge and wild populations. These variations are potentially attributable to different environmental conditions that influenced the refuge and wild populations.

Keywords: Conservation, recovery, morphological change, multivariate morphometrics.

RESUMEN

El pez cachorrito de Sonoyta (*Cyprinodon eremus*) es una especie en peligro de extinción endémica de la cuenca del Río Sonoyta en el noroeste de México y el suroeste de Estados Unidos. Para ayudar en los esfuerzos de conservación de esta especie en México, se estableció una población de refugio en un estanque artificial en 1988 en el Centro Ecológico de Sonora en Hermosillo, Sonora mediante la translocación de individuos de la población del Río Sonoyta. Usamos métodos morfométricos multivariados para delinear las variaciones en la forma del cuerpo en la población del refugio después de 29 años de aislamiento en comparación con individuos silvestres recolectados de la misma muestra. Se observaron variaciones significativas en la forma del cuerpo entre las poblaciones del refugio y las silvestres. Estas variaciones son potencialmente atribuibles a diferentes condiciones ambientales que influyeron en las poblaciones silvestres y del refugio.

Palabras clave: Conservación, recuperación, cambio morfológico, morfometría multivariada.

INTRODUCTION

The Sonoyta pupfish (*Cyprinodon eremus*) (Miller and Fuiman, 1987) is distributed in the Sonoyta River basin and the Quitobaquito Springs in northwestern México and the southwestern United States of America (USA) (Echelle *et al*., 2000; Miller *et al*., 2009; Minckley and Marsh, 2009). The Quitobaquito Springs population is stable, however, the Sonoyta River population is decreasing. These declines are the result of exotic fish introductions, drought, and groundwater pumping that dramatically affects native aquatic habitats (Miller and Fuiman, 1987; Miller *et al*., 2009; Minckley and Marsh, 2009). Currently, this trend of degradation and desiccation continues in the Sonoyta River promoted by the need for water for human growth (Miller *et al*., 2009; Minckley and Marsh, 2009; Minckley *et al*., 2013). The only perennial water flow in the river that persists, is about 1 km in length at the Agua Dulce or Papalote locality (USON 0222, Table 1), and is maintained during the dry season by underground flow of shallow waters (Minckley *et al*., 2013).

C. eremus is considered as endangered by the International Union for Conservation of Nature and the United States government (NatureServe *et al*., 2019). Strategies to manage *Cyprinodon* spp. include protecting their habitat and developing refuge populations to increase or re-establish wild populations in cases of extirpation (Minckley *et al*., 1991; Minckley, 1995; Koike *et al*., 2008). Refuges for *C. eremus* have been established in México to hamper the species' gradual extinction (Minckley *et al*., 2013). These conservation efforts also echo an initiative that sought to recover native fish in the arid southwest during the 1960s (Minckley, 1995). In 1988, the first refuge population was created from the Sonoyta River populations in an artificial pond with only lentic habitat located at Centro Ecológico de Sonora (CES) in Hermosillo, Sonora (Marsh and Sada, 1993). Between 2007 and 2011, another five refuges were established: one in the Biological Station and another in Schuk Toak Visitor Center (translocated to the Biological Station refuge), both of them at El Pinacate y Gran Desierto de Altar Biosphere Reserve (RBEPGDA), one at Centro Intercultural de Estudios de Desiertos y Océanos

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Received: october 20, 2023
Accepted: february 19, 2023
Published: march 4, 2023

DOI: 10.18633/biotecnia.v26.2183

Table 1. Collecting sites for specimens from wild *Cyprinodon eremus* populations used in this study for morphometric and meristic analyses. USON = Universidad de Sonora, Hermosillo, M = Males, F = Females.
Tabla 1. Sitios de recolección de especímenes de poblaciones silvestres de *Cyprinodon eremus* utilizados en este estudio para análisis morfométricos y merísticos. USON = Universidad de Sonora, Hermosillo, M = Machos, F = Hembras.

Species	Locality	Catalog number	Collection date (dd/mm/yy)	Geographic coordinates	Specimens analyzed	
					M	F
C. eremus	Sonora, Sonoyta River Basin, Ejido Josefa Ortíz de Domínguez, at Sonoyta-San Luis Río Colorado highway (km 11).	USON-0148	01 09 1987	31°54'N 112°58'W	17	14
C. eremus	Sonora, Río Sonoyta Basin, El Papalote, around 2 km southwest of Quitobaquito on the Sonoyta River, km 20 of the Sonoyta-San Luis Río Colorado highway.	USON-0222	07 09 1987	31°56'N 113°02'W	10	13
C. eremus	Sonora, Sonoyta River Basin, around 5 km south of the Sonoyta-San Luis Río Colorado highway (km 28).	USON-0225	07 09 1987	31°55.980'N 113°1.980'W	3	3
C. eremus	Sonora, Hermosillo city, Centro Ecológico de Sonora, in an artificial pond,	USON-1386	10 11 2017	29°0.794'N 110°57.059'W	30	30

(CEDO), one at Colegio de Bachilleres in Sonoyta (COBACH), and another at Quitovac that include fish stocked in the springs (Minckley *et al*., 2013). Currently, only the refuges at the RBEPGDA, COBACH, CEDO, and CES remain. The refuge in CES is the most populated (>1000 fish) for *C. eremus* in México. All the refuges were founded in México without an evaluation of their genetic variability.

Previously published papers on the pupfish family (Cyprinodontidae) have reported that populations with 15–30 years of isolation in distinct habitats develop morphological and genetic variations in the translocated populations (Collyer *et al*., 2005; Wilcox and Martin, 2006; Collyer *et al*., 2007; Koike *et al*., 2008; Lema, 2008; Collyer *et al*., 2011; Finger *et al*., 2013; Collyer *et al*., 2015; Black *et al*., 2017). These morphological changes may be attributed to phenotypic plasticity in response to alterations in the environmental conditions of the new habitats (Collyer *et al*., 2005; Wilcox and Martin, 2006; Collyer *et al*., 2007; Lema 2008; Collyer *et al*., 2015; Black *et al*., 2017), including evolutionary processes that occur on an ecological time scale (Collyer *et al*., 2007; Collyer *et al*., 2011). Translocating individuals to be used as reproductive stocks poses a risk for species management due to phenotypic differentiation (Collyer *et al*., 2005; Wilcox and Martin, 2006; Collyer *et al*., 2007; Lema, 2008; Collyer *et al*., 2011) and genetic adaptation to captivity (Frankham, 2008), potentially reducing the survivability of the captive population during reintroduction in a wild environment.

Considering that the Sonoyta pupfish population of the Sonoyta River has decreased drastically and the habitat historical water flow does not exist, the CES refuge population represents an opportunity for the recovery of the species. However, it has been isolated since 1988 in an artificial pond under distinct environmental conditions compared with the wild populations. In this regard, we performed multivariate morphometric analysis (Blackith and Reyment, 1971; Reyment, 1982) to characterize the morphological discrepancies between Sonoyta River wild *C. eremus* populations and CES refuge collected and founded from the same sample, respectively, with the goal of evidence the morphological variation after 29 years of isolation. The results obtained will help in redesigning, increasing the shelter area, and creating future management plans for species conservation.

MATERIAL AND METHODS

Sample collection

Samples of the wild *Cyprinodon eremus* were collected from the Sonoyta River in 1987 using different seines. A subsample was fixed in 10 % formaldehyde and preserved in 50 % ethanol for final deposition in the native fish collection of the Departamento de Investigaciones Científicas y Tecnológicas de la Universidad de Sonora (DICTUS). Another subsample from the wild fish collected in 1987 was kept alive and transported to CES facilities to establish the refuge population in 1988. All subsequent analyses herein were performed with the vouchers of the original collection and the offspring of the founding subsample.

We performed comparative morphometric analysis with 60 adult specimens (30 females and 30 males) of the wild *C. eremus* collected in 1987, as well as 60 adult specimens (30 females and 30 males) from the CES refuge population collected in 2017 using G-Minnow Traps (Table 1).

Morphometric analysis

Based on Hubbs and Lagler (2004) and the box-truss protocol (Strauss and Bookstein, 1982; Bookstein *et al.,* 1985; Table 2; Figure 1), 35 morphological distances were measured considering that these characters underwent variation in both sexes of the genus *Cyprinodon*, as a result of the distinct habitats (Humphries *et al.,* 1981; Collyer *et al.,* 2005; 2015; Black *et al.,* 2017). Seven meristic characters were also counted (Table 2) based on the description of *C. eremus* by Miller and Fuiman (1987). Females and males were separately analyzed due to

Table 2. Morphological distances modified from Humphries *et al.* (1981), Miller and Fuiman (1987), Hubbs and Lagler (2004), Collyer *et al*. (2005), Collyer *et al.* (2015), Black *et al.* (2017), and additional measures based on the box truss protocol (Strauss and Bookstein, 1982; Bookstein *et al*., 1985) and quantified meristic characters for *Cyprinodon eremus* based on Miller and Fuiman (1987).
Tabla 2. Distancias morfológicas modificadas de Humphries *et al*. (1981), Miller y Fuiman (1987), Hubbs y Lagler (2004), Collyer *et al*. (2005), Collyer *et al.* (2015), Black *et al.* (2017) y medidas adicionales basadas en el protocolo box truss (Strauss y Bookstein, 1982; Bookstein *et al*., 1985) y caracteres merísticos cuantificados para *Cyprinodon eremus* basados en Miller y Fuiman (1987).

Code	Morphometric character
M1-3	Dorsal length of head
M1-23	Upper lip - Center of the eye
M1-17	Head length
M1-12	Preanal length
M1-14	Prepelvic length
M1-16	Ventral length of head
M1-4	Length of upper jaw
M2-23	Lower lip - Center of the eye
M2-10	Standard length
M3-5	Occiput - Dorsal fin origin
M3-14	Occiput - Pelvic fin origin
M3-16	Occiput - Isthmus
M5-7	Length of depressed dorsal fin
M5-6	Length of dorsal fin base
M5-11	Dorsal fin origin - Base of the last anal fin ray
M5-14	Body depth
M5-16	Dorsal fin origin - Isthmus
M6-8	Dorsal length of caudal peduncle
M6-9	Base of the last dorsal fin ray – Ventral base of the caudal fin
M6-11	Anterior depth of caudal peduncle
M6-14	Base of the last dorsal fin ray - Pelvic fin origin
M8-9	Depth of caudal peduncle
M8-11	Dorsal base of the caudal fin - Base of the last anal fin ray
M9-11	Ventral length of caudal peduncle
M11-14	Base of the last anal fin ray – Pelvic fin origin
M12-13	Length of depressed anal fin
M14-15	Length of pelvic fin
M14-16	Pelvic fin origin - Isthmus
M18-19	Length of pectoral fin
M18-20	Length of pectoral fin base
M21-22	Eye diameter
A1	Interorbital width
A2	Head width
A3	Width of gape
A4	Body width
No.	**Meristic character**
1	Dorsal fin rays
2	Anal fin rays
3	Pectoral fin rays
4	Pelvic fin rays
5	Caudal fin rays
6	Scales from the dorsal fin origin to anal fin origin
7	Caudal peduncle scale count

the sexual dimorphism of cyprinodontids. Each specimen was examined with a digital caliper (precision 0.01 mm) connected to a personal computer.

The regression described by Elliot *et al.* (1995) was performed to standardize the body measurements of each specimen. This regression model removes the component of size from the shape measurements (allometry), thereby homogenizing their variances (Jolicoeur, 1963). Each character was standardized using the equation $Ms = Mo\ (Ls/Lt)^b$, where Ms = standardized measurement of the character; Mo = original measurement of the character (mm); Ls = average standard length (mm) of all the specimens from all the examined taxa; Lt = standard length (mm) of the specimen; and, "b" was estimated for each character from the observed data via the nonlinear regression equation $M = aL^b$. The parameter "b" was estimated as the slope of the regression log Mo on log Lt, using data from all specimens. The parameter "a" is the non-standardized measurement of the character (mm) and L = Ls/Lt.

The standardized morphometric data and meristic values of all the specimens were used to perform discriminant function analysis (DFA) and principal component analysis (PCA), which are the most used analyzes in multivariate morphometrics (Humphries *et al*., 1981; Reyment, 1982; Turan, 1999). In the case of the PCA it does not require an *a priori* assignment of individuals into groups, but rather summarizes in linear combinations, called Principal Components, the variables that describe the shape variation in the combined sample (Humphries *et al*., 1981; Turan, 1999). On the DFA, the individuals are assigned *a priori* into groups to calculate the function that better discriminates between the groups (Humphries *et al*., 1981; Turan, 1999). The DFA was performed independently for females and males via a forward stepwise form using Statistica 5.0 software (StatSoft, Inc., Tulsa). It was performed to determine the combination of variables that optimally discriminated between wild and refuge populations. Statistically significant differences between populations were determined using Wilks' lambda (λ), which oscillates from 0.0 (perfect discrimination power) to 1.0 (absence of discrimination). Values with $p < 0.05$ obtained in the DFA were considered statistically significant. The PCA was performed using the "factoextra" (Kassambara and Mundt, 2020) and "FactoMineR" (Lê *et al*., 2008) R packages (R Core Team, 2021) to determine which morphological variables best explained the variability in the dataset.

The most important morphological characters selected by DFA and PCA were illustrated by violin and box plots. A one-way analysis of variance with a 95 % confidence interval was performed for each character to evaluate the null hypothesis of equality between the populations. After the null hypothesis was rejected, a post-hoc Tukey test was performed on Statistica 5.0 software (StatSoft, Inc., Tulsa) to verify whether the groups significantly differed (Turan, 1999).

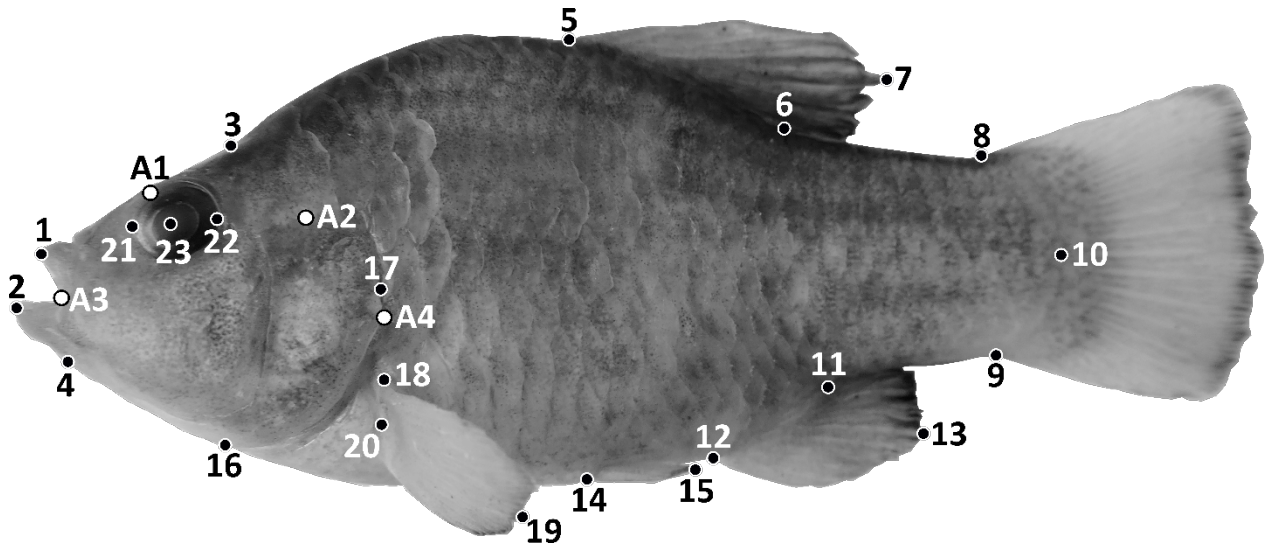

Fig. 1. Landmarks for Box Truss protocol used in *Cyprinodon eremus* analysis. Black dots represent landmarks for distance measurements and the white dots represent the reference marks for the width measurements (Table 2 contains explanation of measurement codes).
Fig. 1. Marcas para el protocolo Box Truss utilizado en el análisis de *Cyprinodon eremus*. Los puntos negros representan puntos de referencia para las medidas de distancia y los puntos blancos representan las marcas de referencia para las medidas de ancho (Tabla 2 contiene explicación de los códigos de medición).

RESULTS

DFA was performed on 120 specimens of *C. eremus*, females and males, from wild and refuge populations. 17 of the 41 morphological and meristic characters among females significantly distinguished the two populations (Table 3). The overall Wilks' lambda (λ) was 0.06404 ($p < 0.0001$), indicating a high degree of discrimination between the two female populations. A significant difference was observed for eight variables (Table 3). According to PCA for wild and refuge females, principal components 1 and 2 combined explained 38.656% of the total variance, the PC1 and PC2 explained 25.329 % and 13.327 %, respectively (Supplementary Table 1).

The scatterplot shows segregation between wild and refuge females, mostly along PC1 (Figure 2A). The variables that most contributed to PC1 were body depth, base of the last dorsal fin ray to pelvic fin origin, dorsal fin origin to base of the last anal fin ray, dorsal fin origin to isthmus, body width, anterior depth of caudal peduncle, depth of caudal peduncle, and occiput to pelvic fin origin, among others (Figure 2B; Supplementary Table 2). Of these, only body depth was selected by the DFA, and it was slightly non-significant ($p = 0.0565$, Table 3). In addition, the DFA selected the length of dorsal fin base, occiput to isthmus, length of pectoral fin base, length of the upper jaw, and width of gape to significantly ($p < 0.05$) discriminate between groups (Table 3); these variables also contributed to PC1 in the PCA (Figure 2B; Supplementary Table 2). Conversely, head width, caudal fin rays, and pectoral fin rays were significant ($p < 0.05$) in the discriminant function (Table 3) but contributed least to PC1 in the PCA (Supplementary Table 2).

The DFA selected 25 morphological and meristic variables that best discriminated the two male populations (Table 4). As observed in females, a high degree of discrimination between both populations was observed based on the Wilks' lambda ($\lambda = 0.08582$, $p < 0.0001$). Significant differences were observed in 13 variables ($p < 0.05$) (Table 4). The first two PCs in the PCA explained 31.87 % of the male variability, where PC1 explained 19.46 % and PC2 12.41% of the variance (Supplementary Table 3). The variables that contributed more to the PC1 were body depth, dorsal fin origin to the base of the last anal fin ray, occiput to isthmus, head length, depth of caudal peduncle, dorsal length of head, and occiput to pelvic fin origin (Figure 3B; Supplementary Table 4). However, the two *C. eremus* male populations were mostly differentiated along PC2 (Figure 3A), where the variables that most contributed to this PC were head width, the width of gape, lower lip to the center of the eye, base of the last dorsal fin ray to pelvic fin origin, base of the last anal fin ray to pelvic fin origin, eye diameter, length of upper jaw, preanal length, and anterior depth of caudal peduncle, among others (Figure 3B; Supplementary Table 4). Of these variables, the head width, base of the last dorsal fin ray to pelvic fin origin, lower lip to center of the eye, length of upper jaw and anterior depth of caudal peduncle were also significant ($p < 0.05$) in the DFA.

Other key variables in both the DFA ($p < 0.05$) and PC2 of the PCA were ventral length of head, head length, length of depressed dorsal fin and length of depressed anal fin (Table 4; Supplementary Table 4). However, the body depth, base of the last dorsal fin ray to ventral base of the caudal fin, scales from the dorsal fin origin to anal fin origin, and dorsal fin rays were important in the DFA ($p < 0.05$) (Table 4) but contributed less to PC2 of the PCA (Supplementary Table 4).

Finally, the most significant morphometric variables in the DFA that contributed most to the PCA in both female and male populations, were plotted using violin and box plots (Figure 4). There were 19 divergent morphometric characters between wild and refuge *C. eremus* populations. Both sexes

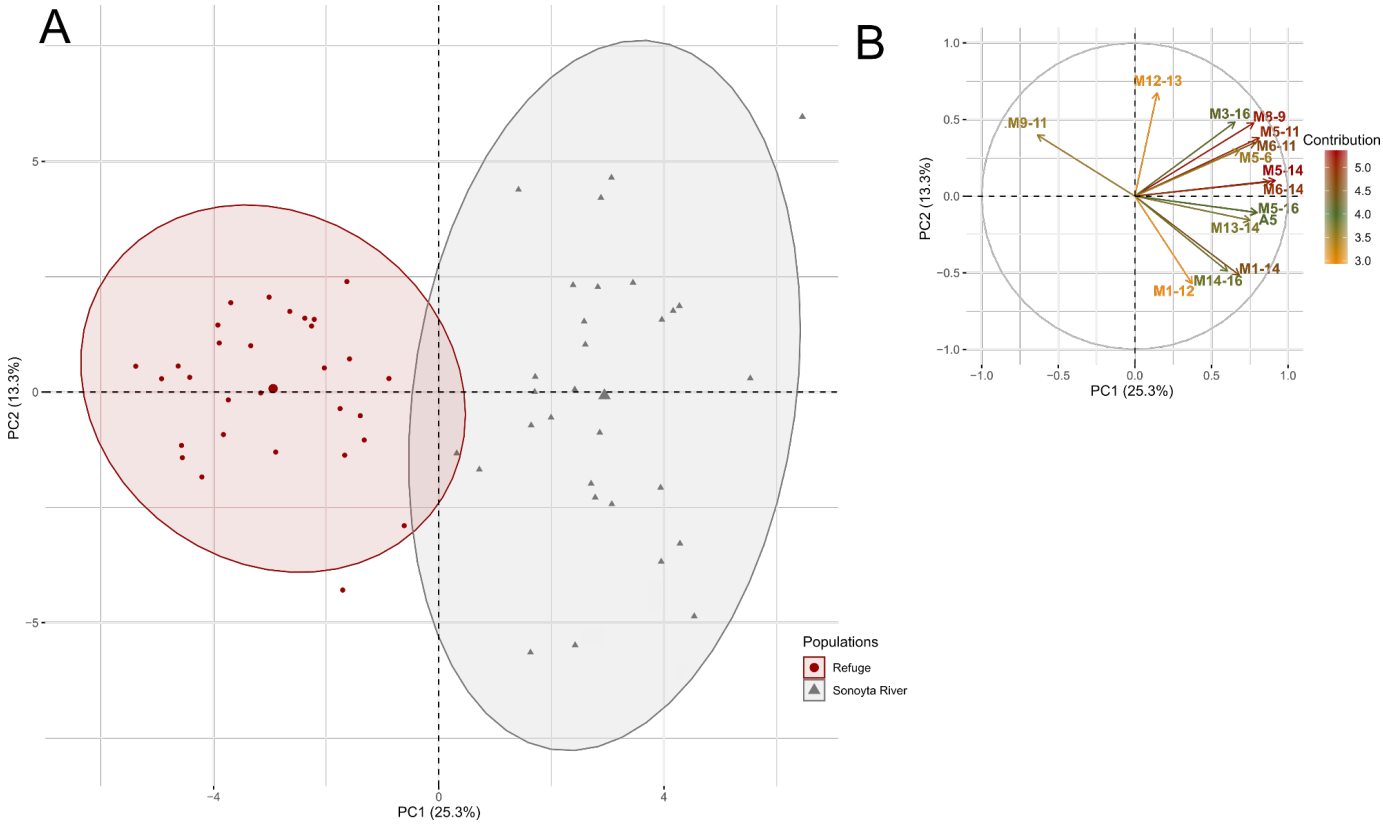

Fig. 2. PCA for the two *C. eremus* females' populations: (A) Scatterplots showing the position of the females along the first two PCs, the ellipses represent the 0.95 confidence intervals; (B) the correlation circle of the 15 variables that most contribute to these PCs (See supplementary table 2 for more information about the contribution of the variables).

Fig. 2. ACP para las dos poblaciones de hembras de *C. eremus*: (A) Gráfico de dispersión mostrando la posición de las hembras en los dos primeros CPs, las elipses representan los intervalos de confianza de 0.95; (B) círculo de correlación de las 15 variables que más contribuyen a estos CPs (Ver tabla suplementaria 2 para más información acerca de la contribución de las variables).

Table 3. Discriminant function analysis summary and the standardized coefficients in the discriminant function for the two *C. eremus* females' populations analyzed. Wilks' lambda values, significance (*p*) and tolerance for 17 variables selected by forward stepwise discriminant function analysis. Wilks' lambda: 0.06404 ($p < 0.0001$). Significant variables ($p < 0.05$) are indicated in bold.

Resumen del análisis de función discriminante y los coeficientes estandarizados en la función discriminante para las dos poblaciones de hembras de *C. eremus* analizadas. Valores lambda de Wilks, significancia (*p*) y tolerancia para 17 variables seleccionadas mediante análisis de función discriminante paso a paso hacia adelante. Lambda de Wilks: 0.06404 ($p < 0.0001$). Las variables significativas ($p < 0.05$) se indican en negrita.

Character	Wilks' Lambda	Partial Lambda	F-remove (1,42)	p-value	Tolerance	Coefficient
Body depth	0.0699	0.9161	3.8471	0.0565	0.6126	0.3825
Length of upper jaw	**0.0878**	**0.7290**	**15.6142**	**0.0003**	**0.6940**	**-0.6459**
Length of dorsal fin base	**0.0816**	**0.7844**	**11.5455**	**0.0015**	**0.4781**	**0.6942**
Occiput – Dorsal fin origin	0.0680	0.9420	2.5844	0.1154	0.6579	0.3068
Caudal fin rays	**0.0857**	**0.7474**	**14.1921**	**0.0005**	**0.6103**	**0.6649**
Length of pectoral fin base	**0.0777**	**0.8245**	**8.9375**	**0.0047**	**0.5543**	**0.5815**
Head width	**0.0896**	**0.7145**	**16.7814**	**0.0002**	**0.5088**	**-0.7742**
Occiput – Isthmus	**0.0708**	**0.9051**	**4.4027**	**0.0419**	**0.4871**	**0.4562**
Pectoral fin rays	**0.0786**	**0.8144**	**9.5727**	**0.0035**	**0.5427**	**-0.6045**
Pelvic fin rays	0.0668	0.9592	1.7884	0.1883	0.6991	0.2498
Scales from the dorsal fin origin to anal fin origin	0.0667	0.9595	1.7747	0.1900	0.8171	0.2302
Width of gape	**0.0703**	**0.9107**	**4.1191**	**0.0488**	**0.6585**	**0.3807**
Upper lip – Center of the eye	0.0687	0.9318	3.0719	0.0870	0.5238	-0.3729
Preanal length	0.0687	0.9324	3.0466	0.0882	0.5644	0.3578
Dorsal fin rays	0.0675	0.9489	2.2614	0.1401	0.6994	-0.2794
Head length	0.0673	0.9511	2.1603	0.1491	0.3735	0.3741
Dorsal length of head	0.0664	0.9650	1.5252	0.2237	0.3722	-0.3171

Table 4. Discriminant function analysis summary for males and the standardized coefficients in the discriminant function for the two *C. eremus* populations analyzed. Wilks' lambda values, significance (*p*) and tolerance for 18 variables selected by forward stepwise discriminant function analysis. Wilks' lambda: 0.08582 ($p < 0.0001$). Significant variables ($p < 0.05$) are indicated in bold.
Tabla 4. Resumen del análisis de función discriminante para los machos y los coeficientes estandarizados en la función discriminante para las dos poblaciones de *C. eremus* analizadas. Valores lambda de Wilks, significancia (*p*) y tolerancia para 35 variables seleccionadas mediante análisis de función discriminante paso a paso hacia adelante. Lambda de Wilks: 0.08582 ($p < 0.0001$). Las variables significativas ($p < 0.05$) se indican en negrita.

Character	Wilks' Lambda	Partial Lambda	F-remove (1,34)	p-value	Tolerance	Coefficient
Anterior depth of caudal peduncle	**0.0984**	**0.8725**	**4.9683**	**0.0325**	**0.2455**	**-0.7537**
Head width	**0.0984**	**0.8725**	**4.9670**	**0.0326**	**0.3131**	**0.6673**
Length of depressed anal fin	**0.1392**	**0.6166**	**21.1438**	**0.0001**	**0.2658**	**-1.2562**
Scales from the dorsal fin origin – Anal fin origin	**0.0995**	**0.8621**	**5.4390**	**0.0258**	**0.5077**	**0.5451**
Occiput – Pelvic fin origin	0.0866	0.9906	0.3243	0.5728	0.2268	0.2135
Length of upper jaw	**0.1038**	**0.8269**	**7.1179**	**0.0116**	**0.3876**	**0.6990**
Base of the last dorsal fin ray – Ventral base of the caudal fin	**0.1567**	**0.5476**	**28.0904**	**0.0000**	**0.1339**	**1.9228**
Dorsal fin rays	**0.1030**	**0.8330**	**6.8176**	**0.0133**	**0.2861**	**0.7991**
Dorsal base of the caudal fin – Base of the last anal fin ray	0.0866	0.9911	0.3050	0.5844	0.2801	-0.1863
Ventral length of caudal peduncle	0.0902	0.9516	1.7302	0.1972	0.2896	-0.4277
Base of the last dorsal fin ray – Pelvic fin origin	**0.1094**	**0.7847**	**9.3293**	**0.0044**	**0.1794**	**1.1457**
Prepelvic length	0.0937	0.9158	3.1244	0.0861	0.3318	0.5268
Dorsal fin origin – Isthmus	0.0908	0.9447	1.9893	0.1675	0.3914	-0.3930
Lower lip – Center of the eye	**0.0976**	**0.8791**	**4.6758**	**0.0377**	**0.1683**	**-0.8864**
Caudal peduncle scale count	0.0928	0.9250	2.7582	0.1060	0.4560	-0.4243
Body depth	**0.1119**	**0.7666**	**10.3489**	**0.0028**	**0.0616**	**-2.0361**
Ventral length of head	**0.1171**	**0.7331**	**12.3770**	**0.0013**	**0.1500**	**1.3949**
Length of depressed dorsal fin	**0.1004**	**0.8551**	**5.7592**	**0.0220**	**0.1594**	**-0.9971**
Dorsal length of caudal peduncle	0.0904	0.9495	1.8066	0.1878	0.3103	-0.4217
Pectoral fin rays	0.0892	0.9616	1.3575	0.2521	0.5318	0.2810
Head length	**0.0969**	**0.8855**	**4.3955**	**0.0435**	**0.1488**	**0.9174**
Width of gape	0.0940	0.9128	3.2480	0.0804	0.1939	-0.7014
Base of the last anal fin ray – Pelvic fin origin	0.0889	0.9649	1.2364	0.2740	0.4171	-0.3033
Pelvic fin origin – Isthmus	0.0903	0.9507	1.7642	0.1929	0.3970	0.3687
Caudal fin rays	0.0892	0.9624	1.3279	0.2572	0.5229	-0.2804

of the refuge population exhibited a greater length of the upper jaw but a shorter body width, length of the depressed dorsal fin, dorsal fin origin to the base of the last anal fin ray, anterior depth of the caudal peduncle, dorsal fin origin to the isthmus, and base of the last dorsal fin ray to pelvic fin origin compared with the wild population (Figure 4 A-G).

The females of the refuge population had a reduced width of gape, lower lengths from the occiput to pelvic fin origin and from the occiput to the isthmus, lower depths of body and caudal peduncle, and shorter length of the dorsal and pectoral fin bases compared with those of the wild *C. eremus* population (Figure 4 H-N, respectively). Conversely, the males of the refuge population showed greater widths of the gape (Figure 4 H) and of the head, higher ventral length of head, and preanal length, but a shorter length from the base of the last anal fin ray to pelvic fin origin and shorter length of depressed anal fin compared with those of the wild *C. eremus* population (Figure 4 O-S).

DISCUSSION

The present study showed evidence of morphological variations in the refuge population of *C. eremus*, after 29 years of isolation in an artificial pond with a homogeneous environment distinct from its wild habitat. Upon comparing the CES refuge population with the wild population originally collected in a natural stream habitat from the Sonoyta River during the establishment of the refuge, we detected morphotypes associated with habitat type. Changes were observed in the mouth, head, body, and caudal peduncle regions. The refuge population had a longer upper jaw and

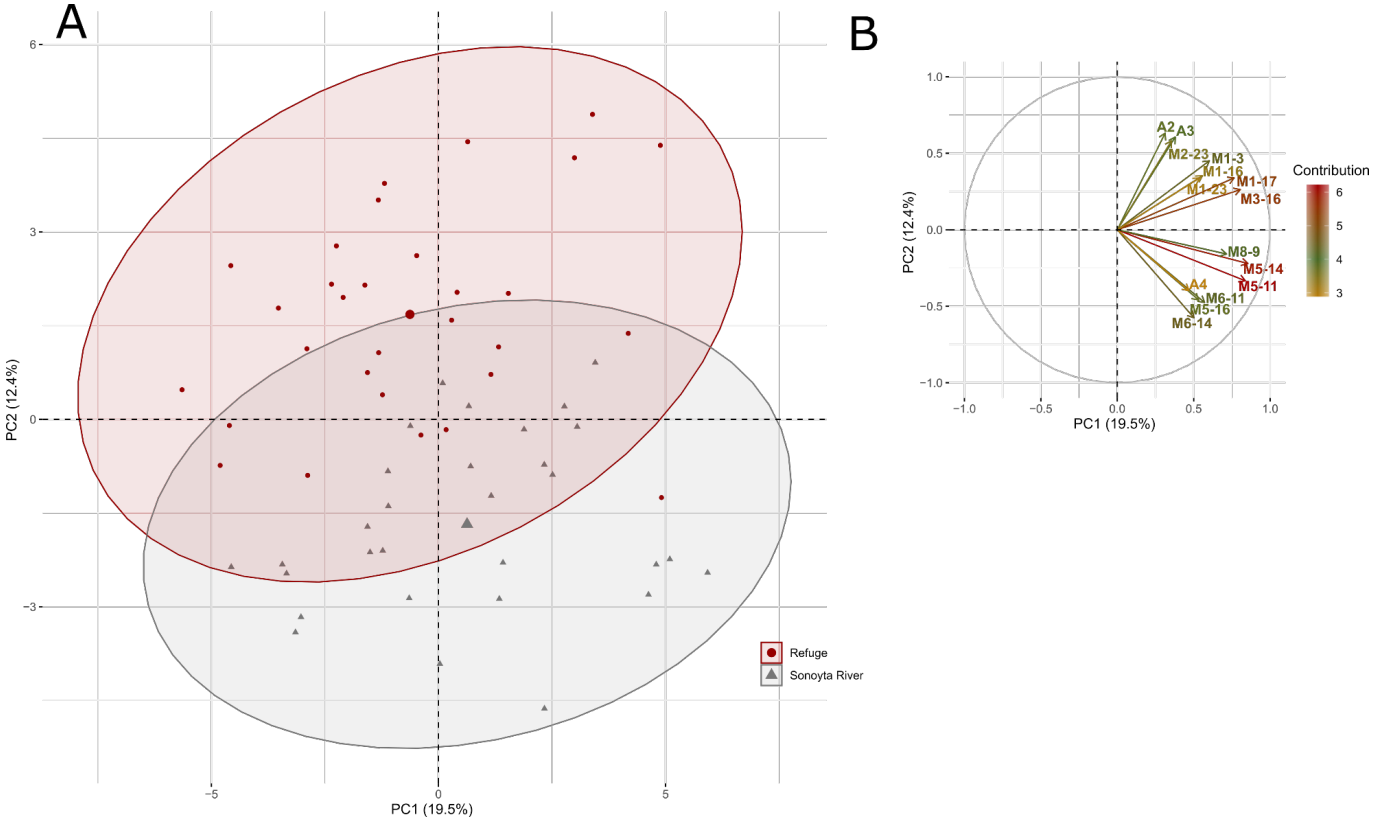

Fig. 3. PCA for the two *C. eremus* males' populations: (A) Scatterplots showing the position of the males along the first two PCs, the ellipses represent the 0.95 confidence intervals; (B) the correlation circle of the 15 variables that most contribute to these PCs (See supplementary table 4 for more information about the contribution of the variables).

Fig 3. ACP para las dos poblaciones de machos de *C. eremus*: (A) Gráfico de dispersión mostrando la posición de los machos en los dos primeros CPs, las elipses representan los intervalos de confianza de 0.95; (B) círculo de correlación de las 15 variables que más contribuyen a estos CPs (Ver tabla suplementaria 4 para más información acerca de la contribución de las variables).

varied width of gape. McGee *et al.* (2013) suggested that jaw traits affect feeding kinematics in fishes. Changes in head orientation and upward repositioning have been detected in Devil's Hole pupfish *Cyprinodon diabolis* (Wilcox and Martin, 2006) and *Cyprinodon bovinus* (Black *et al*., 2017), which may be related to foraging behavior (Black *et al*., 2017). Furthermore, changes in body depth and width were observed in the refuge *C. eremus* population. Similar variations observed in *Cyprinodon pecosensis* have been related to the size of their intestine due to the different types of food available in their habitat (Collyer *et al*., 2015). The distributions and types of food available in the water column likely differ between the wild and refuge habitats of *C. eremus*. Ultimately, these variables may contribute to morphological changes observed in the wild and refuge populations.

The refuge *C. eremus* males had wider heads. Previously, *C. pecosensis* found in lentic sinkhole populations exhibited longer heads, which was attributed to a larger gill size adapted to prevent hypoxia in a low dissolved oxygen environment (Collyer *et al*., 2015). Here, the *C. eremus* refuge population inhabits an artificial pond with limited water circulation and the presence of algae. These factors may contribute to a wider head, that allows more gill space, thereby reducing the risk of hypoxia when the dissolved oxygen levels in the refuge pond decrease.

Modifications in the pectoral fin attachment have been associated with enhanced maneuverability in the water column (Black *et al*., 2017). Moreover, the reduction in the length of the pectoral fin base in *C. eremus* refuge females, depressed dorsal fin in refuge individuals, depressed anal fin in refuge males, and base of the dorsal fin in refuge females may be associated with a lower requirement for stability in the artificial pond, an environment without running water. Also, the anterior depth of the caudal peduncle was lower in both females and males from the refuge population; in the refuge females, the length from the base of the last dorsal fin ray to pelvic fin origin, was shorter, and the depth of the caudal peduncle was lower accounting for slender caudal regions. Variations in the caudal region of *Cyprinodon* have been associated with water flow and the presence of predators (Tobler and Carson, 2010; Collyer *et al*., 2015). Thus, individuals from the refuge population, especially females, had slender caudal regions, potentially because they did not need to swim against the watercourse or move between habitats in the lentic pond environment.

Although morphological differences were observed between the wild and refuge *C. eremus* males, more disparities were observed between females between these populations. In *C. diabolis,* wild males have been shown to be more ag-

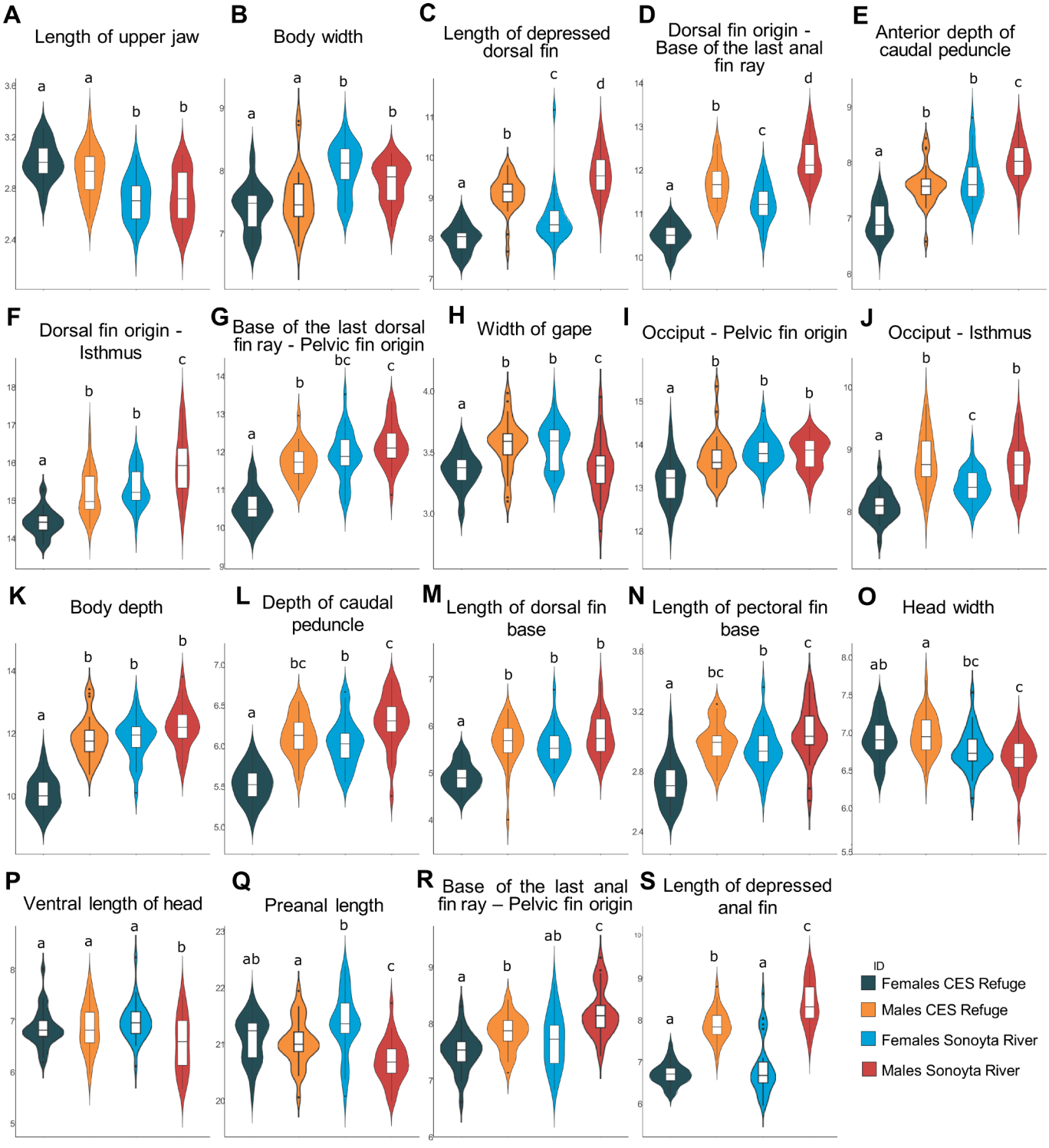

Fig 4. Violin and box plots of the most notable morphological characters for differentiating wild and refuge populations of Cyprinodon eremus. The letters above the bars represent the significant differences among groups according to the Tukey test ($p < 0.05$), and the Y-axis values are in millimeters.
Fig 4. Gráficos de violín y diagramas de cajas de los caracteres morfológicos más destacados para diferenciar poblaciones silvestres y de refugio de Cyprinodon eremus. Las letras sobre las barras representan las diferencias significativas entre grupos de acuerdo con la prueba de Tukey ($p < 0.05$) y los valores en el eje Y están dados en milímetros.

gressive than two populations stocked in two artificial ponds in defending their respective territories (Wilcox and Martin, 2006). Similarly, *C. eremus* males fight each other to protect their territory and reproduce with receptive females (Cox, 1966). Unlike in *C. diabolis* males, within the mechanisms operating in wild *C. eremus* males for intimidating opponents and courting females, the male body shape plays an essential role, which is retained in the refuge males. Refuge males that maintained a body shape similar to wild males likely exhibited better fitness if the selective pressures of the environment were not strong enough to determine survival. Similar results were found in *Cyprinodon tularosa*, wherein males showed a positive association between body depth and size, likely related to the territorial defense, and females showed a decreased association between body depth and size (Collyer *et al*., 2005). Therefore, the morphological variations in *C. eremus* females were more pronounced than in males, because morphological character selection in females may be regulated by environmental conditions and not by sexual selection.

Rodríguez-Ramírez *et al*. (2023) recently studied the genetic variability of the CES and other two refuge populations and wild *C. eremus* from the Sonoyta River using seven microsatellite loci. The CES population showed less genetic variability compared to the others. This lower genetic variation in CES refuge is more related to the time of isolation in contrast to the others analyzed (Rodríguez-Ramírez *et al*., 2023). As mentioned by Koike *et al*. (2008) and Finger *et al*. (2013), typical long established pupfish refuge populations showed low diversity and significant divergence in allele frequencies.

Notably, lower genetic variability may reduce the survivability of the CES refuge *C. eremus* population and its ability to reproduce in its native environmental, as observed for other *Cyprinodon* spp. (Wilcox and Martin, 2006; Collyer *et al*., 2011). This could hinder attempts to re-establish or increase native pupfish populations in the Sonoyta River, as has been the case for other Cyprinodontidae species (Hendrickson and Brooks, 1991; Black *et al*., 2017). Therefore, it is necessary to evaluate the phenotypic and genetic diversities of the remaining wild populations and the rest of the refuges, including CES, to detect isolation-induced morphological and genetic variations. Consequently, a management plan is necessary for the conservation of *C. eremus*, considering the information on morphology and genetic variation. Measures must be taken to avoid morphological changes by increasing the heterogeneity of artificial habitats conditions rendering them more similar to wild habitats conditions (Black *et al*., 2017). Wild individuals should also be translocated to the refuge population to increase genetic variability and therefore reduce the decline in fitness (Wilcox and Martin, 2006; Araki *et al*., 2007; Frankham, 2008; Black *et al*., 2017; Rodríguez-Ramírez *et al*., 2023). We recommend an extensive survey to obtain samples from all localities and habitat types of the entire *C. eremus* distribution to account for the phenotypic variability in each habitat.

ACKNOWLEDGMENTS

We thank Centro Ecológico de Sonora for specimens donation to conduct the analysis. To the Colección de Peces Nativos del Laboratorio de Vertebrados e Invertebrados of DICTUS for providing specimens of wild populations of the Sonoyta pupfish. CONAPESCA issuing the Permiso de Pesca de Fomento PPF/DGOPA-023/18 for the collection of specimens. We thanks to the two anonymous referees and the Associated Editor of the Journal for the comments and editions to improve the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY INFORMATION

Supplementary table 1. Principal Components Analysis summary for *C. eremus* females. All the Principal Components are shown, their eigenvalues, percentage of variance and cumulative percentage of variance.

Tabla suplementaria 1. Resumen del Análisis de Componentes Principales para las hembras *C. eremus*. Se muestran todos los Componentes Principales, sus autovalores, porcentaje de varianza y porcentaje de varianza acumulada.

Principal Component	Eigenvalue	Percentage of variance	Cumulative percentage of variance
Principal Component 1	10.385	25.330	25.330
Principal Component 2	5.464	13.327	38.657
Principal Component 3	3.291	8.026	46.683
Principal Component 4	2.856	6.965	53.648
Principal Component 5	1.841	4.489	58.138
Principal Component 6	1.734	4.228	62.366
Principal Component 7	1.526	3.722	66.087
Principal Component 8	1.372	3.346	69.433
Principal Component 9	1.206	2.942	72.374
Principal Component 10	1.058	2.581	74.955
Principal Component 11	1.016	2.478	77.433
Principal Component 12	0.939	2.290	79.723
Principal Component 13	0.864	2.107	81.830
Principal Component 14	0.782	1.907	83.738
Principal Component 15	0.696	1.698	85.436
Principal Component 16	0.649	1.583	87.019
Principal Component 17	0.597	1.455	88.474
Principal Component 18	0.522	1.273	89.747
Principal Component 19	0.489	1.193	90.940
Principal Component 20	0.463	1.130	92.070
Principal Component 21	0.395	0.962	93.032
Principal Component 22	0.381	0.929	93.961
Principal Component 23	0.287	0.699	94.660
Principal Component 24	0.272	0.663	95.323
Principal Component 25	0.261	0.636	95.960
Principal Component 26	0.234	0.570	96.530
Principal Component 27	0.229	0.559	97.089
Principal Component 28	0.185	0.452	97.541
Principal Component 29	0.171	0.417	97.958
Principal Component 30	0.159	0.388	98.346
Principal Component 31	0.124	0.303	98.649
Principal Component 32	0.118	0.287	98.936
Principal Component 33	0.096	0.235	99.171
Principal Component 34	0.065	0.160	99.330
Principal Component 35	0.064	0.156	99.486
Principal Component 36	0.060	0.146	99.632
Principal Component 37	0.049	0.118	99.750
Principal Component 38	0.038	0.093	99.843
Principal Component 39	0.036	0.087	99.930
Principal Component 40	0.020	0.048	99.978
Principal Component 41	0.009	0.022	100

Supplementary table 2. Contribution of each variable to the first five Principal Components in the PCA for *C. eremus* females.
Tabla suplementaria 2. Contribución de cada variable a los primeros cinco Componentes Principales en el ACP para las hembras de *C. eremus*.

Character	PC1	PC2	PC3	PC4	PC5
Dorsal length of head	0.0087	1.2287	14.5306	1.0073	1.2437
Upper lip – Center of the eye	0.1905	1.4202	7.4826	2.2152	0.2027
Head length	0.0000	3.0259	9.8181	5.5030	0.0445
Preanal length	1.3449	5.9523	1.1118	4.2622	3.1700
Prepelvic length	4.4698	4.8511	0.3855	2.9661	0.3140
Ventral length of head	0.3864	1.9233	5.4001	0.7780	6.4097
Length of upper jaw	3.2733	1.3930	1.1742	4.4196	0.1827
Lower lip – Center of the eye	0.1568	2.2097	7.1520	1.1069	0.1734
Occiput – Dorsal fin origin	1.9476	1.6739	0.1469	1.0430	7.8714
Occiput – Pelvic fin origin	5.4459	0.4527	0.8304	3.7536	0.0443
Occiput – Isthmus	4.1115	4.2722	0.6140	0.8561	0.2164
Length of depressed dorsal fin	1.8243	3.8582	0.0020	3.1207	1.1533
Length of dorsal fin base	4.4878	1.6547	0.0172	7.1377	0.1156
Dorsal fin origin – Base of the last anal fin ray	6.3516	2.6368	1.3475	0.0135	0.0032
Body depth	8.0888	0.1881	1.0052	0.0186	0.2613
Dorsal fin origin – Isthmus	6.1461	0.2131	0.0133	0.4343	1.4010
Dorsal length of caudal peduncle	2.2670	2.7064	5.6126	3.5229	0.1246
Base of the last dorsal fin ray – Ventral base of the caudal fin	0.0352	7.8507	3.5532	4.1896	0.0161
Anterior depth of caudal peduncle	5.9668	2.2485	2.1717	0.3784	0.2205
Base of the last dorsal fin ray – Pelvic fin origin	7.4653	0.1589	0.9335	0.0839	0.5642
Depth of caudal peduncle	5.8106	4.1835	0.0066	0.3976	0.0002
Dorsal base of the caudal fin – Base of the last anal fin ray	0.0042	8.2923	4.3005	3.5591	6.2474
Ventral length of caudal peduncle	3.8866	2.9026	1.0788	1.6713	7.6280
Base of the last anal fin ray – Pelvic fin origin	0.6382	0.0129	1.3122	3.5401	6.3243
Length of depressed anal fin	0.2042	8.2842	0.0189	2.9811	0.9898
Length of pelvic fin	0.3302	6.0794	3.8599	0.0000	6.1808
Pelvic fin origin – Isthmus	3.5141	4.3903	0.0218	2.9247	1.4048
Length of pectoral fin	0.5195	3.7250	6.8255	1.7178	0.2353
Length of pectoral fin base	3.9349	0.2714	0.3357	0.0246	0.4428
Eye diameter	0.4816	0.2607	11.9743	1.1347	0.3070
Interorbital width	2.2774	0.0023	0.1211	4.0871	13.6219
Head width	0.1755	4.6747	0.8143	2.1701	9.5027
Width of gape	2.4212	2.8997	1.0667	0.9393	1.4473
Body width	6.0851	0.2059	1.0624	1.0207	1.7812
Scales from the dorsal fin origin to anal fin origin	1.4424	0.9295	0.0357	0.0619	4.3412
Caudal peduncle scale count	2.4048	0.5725	0.1872	1.1563	1.4176
Dorsal fin rays	0.1960	0.0024	0.0153	1.0788	2.0977
Anal fin rays	0.0279	0.1543	1.8445	8.9752	3.5809
Pectoral fin rays	0.2641	0.1019	0.5696	0.1164	7.0393
Pelvic fin rays	0.0000	1.6501	0.1131	15.3607	1.6023
Caudal fin rays	1.4133	0.4861	1.1336	0.2718	0.0747

Supplementary table 3. Principal Components Analysis summary for *C. eremus* males. All the Principal Components are showed, their eigenvalues, percentage of variance and cumulative percentage of variance.
Tabla suplementaria 3. Resumen del Análisis de Componentes Principales para los machos *C. eremus*. Se muestran todos los Componentes Principales, sus autovalores, porcentaje de varianza y porcentaje de varianza acumulada.

Principal Component	Eigenvalue	Percentage of variance	Cumulative percentage of variance
Principal Component 1	7.978	19.459	19.459
Principal Component 2	5.088	12.410	31.869
Principal Component 3	3.962	9.664	41.533
Principal Component 4	3.301	8.052	49.585
Principal Component 5	2.518	6.143	55.727
Principal Component 6	1.933	4.715	60.442
Principal Component 7	1.692	4.126	64.568
Principal Component 8	1.530	3.731	68.299
Principal Component 9	1.319	3.217	71.516
Principal Component 10	1.153	2.813	74.329
Principal Component 11	1.077	2.626	76.955
Principal Component 12	0.917	2.237	79.192
Principal Component 13	0.894	2.180	81.372
Principal Component 14	0.859	2.095	83.467
Principal Component 15	0.781	1.905	85.372
Principal Component 16	0.669	1.632	87.004
Principal Component 17	0.605	1.475	88.479
Principal Component 18	0.590	1.438	89.917
Principal Component 19	0.484	1.181	91.099
Principal Component 20	0.391	0.953	92.052
Principal Component 21	0.379	0.924	92.976
Principal Component 22	0.344	0.839	93.815
Principal Component 23	0.331	0.808	94.623
Principal Component 24	0.323	0.788	95.411
Principal Component 25	0.297	0.724	96.135
Principal Component 26	0.214	0.521	96.656
Principal Component 27	0.199	0.484	97.141
Principal Component 28	0.183	0.446	97.587
Principal Component 29	0.157	0.384	97.971
Principal Component 30	0.141	0.345	98.316
Principal Component 31	0.122	0.298	98.614
Principal Component 32	0.108	0.263	98.877
Principal Component 33	0.097	0.236	99.112
Principal Component 34	0.091	0.223	99.335
Principal Component 35	0.063	0.153	99.488
Principal Component 36	0.057	0.139	99.627
Principal Component 37	0.050	0.122	99.749
Principal Component 38	0.037	0.091	99.840
Principal Component 39	0.034	0.084	99.924
Principal Component 40	0.019	0.047	99.971
Principal Component 41	0.012	0.029	100.000

Supplementary table 4. Contribution of each variable to the first five Principal Components in the PCA for *C. eremus* males.
Tabla suplementaria 4. Contribución de cada variable a los primeros cinco Componentes Principales en el ACP para los machos de *C. eremus*.

Character	PC1	PC2	PC3	PC4	PC5
Dorsal length of head	4.550	3.994	0.419	0.369	0.136
Upper lip – Center of the eye	3.640	2.253	3.467	0.409	6.651
Head length	7.320	2.256	0.004	0.839	2.324
Preanal length	0.339	5.731	2.444	1.653	2.815
Prepelvic length	1.285	2.565	0.397	12.677	1.286
Ventral length of head	3.888	2.449	7.679	0.010	1.710
Length of upper jaw	0.992	5.866	0.144	1.104	1.357
Lower lip – Center of the eye	1.576	6.645	3.489	0.008	4.003
Occiput – Dorsal fin origin	0.043	4.049	0.570	5.510	0.029
Occiput – Pelvic fin origin	4.543	0.071	0.479	6.344	0.160
Occiput – Isthmus	8.123	1.351	0.447	0.093	0.615
Length of depressed dorsal fin	3.509	1.499	1.899	7.500	0.603
Length of dorsal fin base	2.686	0.513	11.391	0.012	1.666
Dorsal fin origin – Base of the last anal fin ray	8.795	2.188	0.628	0.109	0.084
Body depth	9.148	0.957	0.101	1.725	0.962
Dorsal fin origin – Isthmus	3.550	4.139	0.619	4.408	1.801
Dorsal length of caudal peduncle	0.254	0.025	7.065	0.398	13.844
Base of the last dorsal fin ray – Ventral base of the caudal fin	0.682	0.514	10.589	0.744	7.103
Anterior depth of caudal peduncle	4.088	4.443	3.351	0.013	1.016
Base of the last dorsal fin ray – Pelvic fin origin	3.154	6.517	0.465	0.025	0.274
Depth of caudal peduncle	6.409	0.493	0.131	1.145	1.786
Dorsal base of the caudal fin – Base of the last anal fin ray	0.895	0.304	5.803	0.009	9.178
Ventral length of caudal peduncle	0.693	1.406	1.530	0.967	11.585
Base of the last anal fin ray – Pelvic fin origin	0.075	6.373	0.003	1.427	1.126
Length of depressed anal fin	2.366	1.460	0.172	11.487	0.267
Length of pelvic fin	0.454	0.000	4.214	11.650	0.048
Pelvic fin origin – Isthmus	0.015	0.575	4.870	1.695	10.753
Length of pectoral fin	2.876	0.411	1.992	4.929	1.281
Length of pectoral fin base	2.314	0.281	0.416	1.601	1.042
Eye diameter	0.865	5.936	0.709	0.056	0.228
Interorbital width	2.837	0.003	4.184	5.746	3.576
Head width	1.229	7.844	2.066	0.134	1.024
Width of gape	1.802	7.223	3.227	0.461	0.542
Body width	2.753	3.108	0.527	4.127	0.003
Scales from the dorsal fin origin to anal fin origin	1.154	0.254	0.817	0.048	3.709
Caudal peduncle scale count	0.113	2.257	0.650	4.431	0.153
Dorsal fin rays	0.005	0.006	7.409	1.595	0.369
Anal fin rays	0.311	0.059	4.440	0.012	0.213
Pectoral fin rays	0.439	0.001	0.627	0.183	0.888
Pelvic fin rays	0.116	0.029	0.566	0.155	3.761
Caudal fin rays	0.111	3.951	0.001	4.194	0.025

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Biotecnia
vol. 26, e2183, 2024
Universidad de Sonora, División de Ciencias Biológicas y de la Salud,
ISSN-E: 1665-1456

DOI: https://doi.org/10.18633/biotecnia.v26.2183