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Morphological description of gametes in cave and surface populations of *Astyanax mexicanus* (De Filippi, 1853)

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Research Article

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Summary

The Mexican tetra *Astyanax mexicanus* presents two contrasting morphs, a widely distributed surface morph and a cave-adapted morph. These cave-adapted morphs have evolved independently from two different lineages (i.e. 'old' and 'new' lineages); therefore, this model system gives a unique opportunity to explore parallel adaptive evolution in biological traits. The present study corresponds to the first morphological description of the *Astyanax mexicanus* maturation process of the spermatozoa and oocytes, using thermal and hormonal stimuli to promote spermatogenesis and oogenesis, considering surface and cave morphs from both lineages. We corroborate the relevance of thermal and hormonal stimuli to promote gamete maturation. The hormone Ovaprim (GnRHa + Domperidone) is an effective promoter of ovarian development, maturation end in oocytes and spawning in *Astyanax mexicanus*. The sperm morphology of *Astyanax mexicanus* includes the sperm head, the midpiece, and tail or flagellum. We found differences in the spermatozoan total length between environments ($F = 9.929$, $P = 0.05$) and linages ($F = 49.86$, $P = 0.005$). The oocytes showed a spherical conformation with a mean diameter of 822.4 ± 194.1 μm for the surface populations, and 604.6 ± 38.3 μm for the cave populations. The oocyte chorion presents ridges and grooves that are arranged radially towards the micropyle. A plug in the micropyle zone was observed after fertilization, confirmed by the outer membrane of the chorion, which provides some weak adhesiveness to the substrate. We observed differences in chorion thickness between the contrasting environmental conditions. This is the first morphological characterization of the Sótanos Vázquez, Escondido and Tigre, which previous to this study were only known from speleological expeditions, with no previous biological information available.

Introduction

The *Astyanax* genus within the Characidae family is one of the most diverse genera with the largest distribution, and is accompanied by high morphological plasticity, as well as a great capacity to adapt to new environments (Ornelas-García *et al.*, 2008). Possibly one of the most conspicuous examples of this plasticity is the *Astyanax mexicanus* species (Jeffery, 2009), which is endemic to Mexico, presenting two contrasting morphs, a surface and a cave morph, which have proven to be interfertile both under laboratory and wild conditions, making this species a unique model system for evolutionary studies that allow characterization of the genetic bases of morphological change.

Recent explorations have shown that cave morphs are present in at least 31 different cave populations in the Northeast of Mexico, geographically distributed in three mountain ranges in the Sierra del Abra region: Sierra de El Abra, Sierra de Guatemala and Sierra de la Colmena. These cave populations have derived from at least two different surface lineages (Herman *et al.*, 2018), therefore, cave populations have evolved independently. Moreover, phylogenetic analyses of these populations have shown a geographical correspondence between the different mountain ranges and their phylogenetic lineages, therefore the Sierra de El Abra mountain range corresponds to a different lineage (previously known as 'old lineage') to the Sierra de Guatemala and Micos mountain ranges (known as 'new lineage'), each of those cave lineages have their sister surface lineage (please refer to Strecker *et al.*, 2004; Ornelas-García *et al.*, 2008; Hausdorf *et al.*, 2011; Bradic *et al.*, 2012; Strecker *et al.*, 2012; Coghill *et al.*, 2014; Fumey *et al.*, 2018; Herman *et al.*, 2018). In this regard, these populations provide a unique opportunity to compare the adaptations, some regressive and others constructive, to extreme environmental conditions (such as caves), which have originated from an ancestor whose

lineage still exists, which then makes it possible to explore in depth all the biological aspects related to its evolution and adaptation, including the reproduction and gametes maturation under contrasting conditions.

The contrasting conditions between the surface and the caves gave a unique opportunity to explore the adaptations in reproductive biology, particularly in gametes morphology, which could be facing alternative selective pressures. In this regard, previous studies have explored several aspects of the species reproduction, including the environmental factors that can trigger it. Sadoglu (1979) presented a reproduction method based on annual rhythms to obtain regular and frequent spawning, in which he found that *Astyanax mexicanus*, unlike other tropical fish reared in the laboratory, follows an annual reproduction cycle that persists despite the absence of seasonal signals such as variation in the photoperiod and temperature. In addition, within the mating sets he found that the water temperature fluctuated between 19°C and 30°C, however, the optimal water temperature was between 25°C and 28°C with a 72.9% spawning. Recent studies have suggested that abrupt changes in temperature can trigger reproductive behaviour in both morphs (i.e. caves and surface; Borowsky, 2008; Hinaux *et al.*, 2011; Simon *et al.*, 2019), therefore, an abrupt change of +4°C, or a gradual change in +2°C over 3 or 4 consecutive days, from 20°C up to 26°C could promotes spawning. In terms of the photoperiod, most of the protocols report a 12/12 h (light/dark) cycle, however a recent study (Peuß *et al.*, 2019) used a 14/10 h cycle. Most of the previous studies have focused on the effects of temperature on mating behaviour (e.g. Simon *et al.*, 2019), but temperature could be crucial in gamete maturation in characiforms (Pinheiro *et al.*, 2020; Postingel Quirino *et al.*, 2021). In this regard, there are physiological prerequisites for sexual behaviour to occur and result in the fertilization of the spawned eggs: (1) vitellogenesis of oocytes must be completed in the ovary, (2) oocytes must be mature, and ovulated, facilitated by endocrine luteinizing hormone (LH), social and environmental cues, and (3) spermatogenesis must be completed, being important both the amount and quality of the sperm (Crews, 1984; Crews and Moore, 1986; Kobayashi *et al.*, 2002; Munakata and Kobayashi, 2010). Among the previous parameters, ovulation is critical as it determines the occurrence and the timing of sexual behaviour, while the LH surge is determined by environmental conditions such as temperature, photoperiod depth, and water quality, among others (Kobayashi *et al.*, 2002; Munakata and Kobayashi, 2010).

Food is another important factor that can affect species reproduction and growth rate (Izquierdo and Palacios, 2004). In this respect, wild-caught cavefish are well adapted to food scarcity (Hüppop and Wilkens, 1991), therefore, cave animals show selected characteristics compared with surface ones, these characteristics include a slower growth rate, greater longevity, delayed maturity, and fewer but larger eggs (Poulson, 1964; Vandel, 1964; Peck, 1986; Simon *et al.*, 2019). Also, the egg diameters in caves (i.e. Pachón cave) have been reported to be 15% larger than that of the surface fish (Hüppop and Wilkens, 1991). Moreover, previous studies have shown differences among caves regarding yolk volume, with caves with the highest yolk volume after 24 hpf (Hüppop and Wilkens, 1991). Larger eggs with more yolk produce larger embryos, as they can live longer with their food reserves, therefore, caves embryos start external feeding after the 4th and 5th day post fertilization, in contrast with the surface embryos which start the external feeding after the 3rd day (Hüppop, 1988). Conversely, larger larvae are more likely to survive as they exhibit greater mobility to find food or escape from predation (Poulson, 1964; Culver, 1982).

The present study corresponds to the first morphological description of the *Astyanax mexicanus* gametes maturation process of the oocytes and spermatozoa using thermal and hormonal stimuli to promote spermatogenesis and oogenesis, considering both lineages, therefore, for old lineage caves we included the Pachón cave, Sótano Pichijumo and Sótano Tigre; and for the new lineage caves we included Sótano Vázquez, Sótano Escondido and Sótano Caballo Moro. Similarly for the surface we included samples from the two lineages, with Rascón from the old lineage and Bocatoma from the new lineage. Therefore, the main goal of the present study was to characterize the morphological features of gametes (oocytes and spermatozoa) in *Astyanax mexicanus* lineages and characterize their maturation process.

Materials and methods

The surface and cave morphs of the *Astyanax mexicanus* organisms were collected from the wild (Sierra del Abra, Sierra de la Guatemala, and Sierra de la Colmena), under the auspices of the permit SGPA/DGVS/05389/17, delivered by Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT), in which we clearly specified the animal care and use considering SEMARNAT animal welfare laws. The collected fish were transported and kept at the Instituto de Biología, UNAM facility. In total, six cave populations were considered in this study, three from old lineage: Pachón cave, Sótano de Pichijumo, Sótano de Tigre from the old lineage, and three from the new lineage: Sótano de Vázquez, Sótano de Escondido and Sótano de Caballo Moro, and two surface populations: Rascón (old lineage) and Bocatoma (new lineage) (Figure 1). For each population, an experimental unit made up of two males and one female was formed and kept in a 40-litre aquarium (eight in total). Throughout the experiment, the fish were fed five times a day with a mixture of live and frozen brine shrimp (*Artemia salina*), live and frozen blackworm, *Daphnia* sp., dried blood worm, flakes (Wardley) and liver paste. Throughout the experiment we monitored the water quality by measuring the following variables: pH, ammonia (NH_4), nitrite (NO_2), nitrate (NO_3), and phosphate (PO_4), using colorimetric tests (FLUVAL Brand), and conductivity (µs/cm), salinity (PSU) and dissolved oxygen (%) using a HANNA meter (model HI98194). The fish were maintained using 12:12 h, light:dark inverted photoperiod conditions, to promote their reproductive behaviour, which was at their night during our light hours.

Gametes obtention

The mean standard length (SL) of the fish analyzed was 66.23 mm with values of 59–86 mm, with a mean for the caves of a SL of 66.26 mm (59–75 mm), and with a SL mean of 74.76 mm (65–86 mm) for the surface fish. For the new cave lineage, the mean SL value was 70.52 mm (59–75 mm), whereas the old cave lineage mean SL value was 66.26 mm (59–61 mm). The new surface lineage had a mean SL value of 86 mm, while for the surface old lineage the mean SL value was 65 mm.

Spermatozoa

Maturation of male gametes was stimulated randomly by applying four different thermal stimuli, as previously reported

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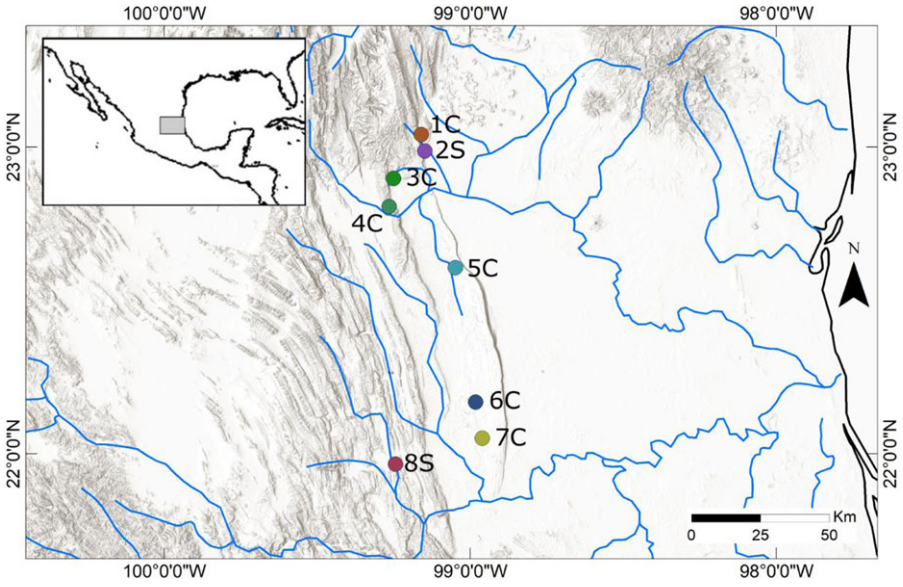

Figure 1. Sampling localities of cave (C) and surface (S) populations of *Astyanax mexicanus* from the Sierra del Abra and Guatemala. 1, Sótano de Escondido; 2, Bocatoma river; 3, Sótano de Caballo Moro; 4, Sótano de Vázquez; 5, Pachón cave; 6, Sótano de Tigre; 7, Pichijumo; and 8, Rascón river.

(Supplementary Material 1), and during the fifth day after the first day applying the thermal treatment. An abdominal massage was performed to obtain the ejaculate (Supplementary Material 2), each fish was anaesthetised with cold water (~7°C). Once the ejaculate was obtained, it was collected with a capillary for a haematocrit and subsequently a smear was produced with the eosin–nigrosin vitality stain.

To perform the smear, the collected ejaculate (2 µl) was placed on a slide and 2 µl of the tank water was added on the ejaculate to activate the sperm and extent its flagellum. It was left to act for 1 min; if it was left longer, the flagellum begins to roll up and becomes superimposed with the flagella of the other sperm, preventing its evaluation. After 1 min had passed, 2 µl of the eosin–nigrosin vitality stain was placed on the mixture (sperm + water) and homogenized with the help of another slide, with which the smear scan was performed, then it was allowed to dry at room temperature (Supplementary Material 3). The slides were observed under an optical microscope with a ×100 objective using an immersion oil and photographs were taken with an optic microscope Olympus IX81, Evolution MP camera, photographs were analyzed using ImageJ software v.1.8.0. The variables measured for each sperm were: total length, head area, and flagellum length, considering a total sample size of per population = 100, except for Pachón ($N = 58$) and Vázquez ($N = 18$), as those were the maximum number of spermatozoa obtained in the samples.

Oocytes

For Sótano de Vázquez and Sótano de Tigre cave populations the oocytes were obtained through abdominal massage after applying thermal stimuli similar to the male's protocol. However, for Pachón, Pichijumo, Escondido, Caballo Moro cave populations and Bocatoma and Rascón surface populations it was necessary to apply a hormonal stimulus. Therefore, to obtain mature oocytes, we needed to apply a hormonal combination of an analogue of salmon gonadotropin-releasing hormone (sGnRHa) and a dopamine inhibitor (Domperidone), commercially known as Ovaprim (Laboratories Syndel, USA). The hormone application was performed following the manufacturer's instructions and considering a dose of 0.5 µl for each gram weight. Our fish had an average weight of 4 g, therefore we applied 2 µl of Ovaprim for each female. As the reference sheet mentioned that this could produce some adverse effects such as necrosis, redness or whitening in the application area, it was decided to dilute the dose with 8 µl of saline solution (SS) giving a final volume of 10 µl. To apply the dose, the fish were first anaesthetised in cold water (~7°C), once anaesthetised, the fish were placed on the palm of the left hand in the right lateral decubitus position and held with the thumb to have greater firmness at the time of the injection, then the dose (10 µl) was applied in the area of the base of their dorsal fin (area with the greatest amount of muscle) placing the needle in the opposite direction to the scales, for this an insulin fine 0.3 ml Caliber 31G × 6 mm ultra-Bd syringe was used (Supplementary Material 2).

After the hormone was applied females spawned ~6 h later, therefore the oocytes were collected and observed under a stereoscopic microscope to see if they were fertilized. All those oocytes were fixed in 0.625% glutaraldehyde solution, in phosphate buffer, pH 7.4. Following fixation, the samples were washed with different concentrations of ethanol until reaching 100%, and dehydrated under CO_2 to a critical point, mounted on stubs, metallized with gold to finally be examined with a scanning electron microscope (SEM) Hitachi model SU1510. Once the photographs were obtained, the images were analyzed using the ImageJ software v.1.8.0 programme to obtain the different measurements of the oocyte. We measured the diameter of the micropyle, as well as the thickness of the chorion, for this, considering the mean of five measures at different points of the chorion.

Statistical analysis

A descriptive statistics table was constructed (i.e. mean, max, min, standard deviation, and standard error) for the spermatozoan measurements. We perform independent *lme* analyses using spermatozoan total length, flagellum length and head area as response variables, and environment and lineage and their interaction as fixed effects predictor variables, using the population as a random

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Table 1. Water quality mean ± standard deviation by week

Week	pH	NO_2	NO_3	NH_4	PO_4	Cond.	Sal.	O_2
1	7.3 ± 0.1	1.1 ± 1.2	9.4 ± 12.4	1.4 ± 1.02	0.9 ± 0.2	439 ± 5.4	0.21 ± 0	35.9 ± 5.9
2	6.8 ± 0.4	2.8 ± 2.4	20.6 ± 13.2	0.7 ± 0.3	0.6 ± 0.2	444.7 ± 7.3	0.21 ± 0.007	28.5 ± 4.2
3	7.1 ± 0.3	1.6 ± 2.2	8.7 ± 5.2	1.9 ± 0.6	0.7 ± 0.3	472.2 ± 7	0.23 ± 0.003	51.1 ± 3.3
4	6.7 ± 0.4	0.1 ± 0.1	5 ± 0	1.5 ± 0.7	0.6 ± 0.3	471.6 ± 15.4	0.23 ± 0.007	23.4 ± 4.2

Cond., conductivity (µs/cm); NH_4, ammonia; NO_2, nitrite; NO_3, nitrate; O_2, dissolved oxygen (%); PO_4, phosphate all of them are expressed in mg/l; Sal., salinity (psu).

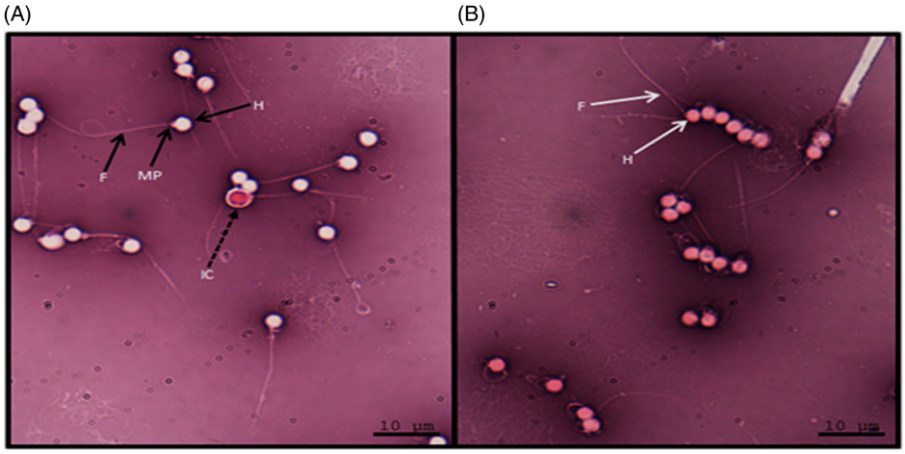

Figure 2. Eosin–nigrosin vitality stain. (A) Dotted arrow points to the immature cell, which is stained pink with a small white halo around the head. Live sperm are shown with the continuous black arrow, H, the sperm head; MP, midpiece; and F, flagellum or tail completely white. (B) Dead sperm, the head (H) is completely stained pink without any halo surrounding it.

effect. Due to the reduced number of observations for the evaluation of the oocytes in the SEM, the values were reported as mean ± standard deviation.

Results

Water quality

We monitored water quality during the gamete's recollection, and the descriptive statistics are presented in Table 1, in which it can be observed that the values of salinity and conductivity increased as time passed, reaching a conductivity of 471.6 µs/cm and salinity of 0.23 psu. While pH values were between 6.7 to 7.3, nitrites and nitrates presented a punctual peak in week 2 (NO_2 = 2.8 mg/l and NO_3 = 20.6 mg/l) but went down after that week. Finally, during 8 weeks we successfully obtained gametes from both sexes.

Spermatozoa

The sperm morphology of *Astyanax mexicanus* was made up of the sperm head, the midpiece, and tail or flagellum (Figure 2). With the eosin–nigrosin vitality stain it was possible to distinguish live spermatozoa from immature and dead cells, in which the immature cells could be identified by presenting the rolled tail (Figure 2A), while the dead cells showed a complete staining of the head with the eosin–nigrosin stain (Figure 2B). Based on our analyses, the populations with the highest percentage of spermatozoa viability were Rascón river (99.3%) and Sótano de Escondido (97.6%), followed by Sótano de Caballo Moro, Sótano de Pichijumo and Sótano de Tigre with 81.5%, 69.1% and 65.8% respectively, while Bocatoma river was the one with the lowest spermatozoa viability (19%). Finally, Sótano de Vázquez males presented the largest percentage of immature spermatozoa (95%), however, those were the males with the lowest SL.

In total, 350 spermatozoa were measured for the spermatozoa total length, flagellum length and head area, 150 from the new lineage (100 from the cave and 50 from the surface), and 200 from the old lineage (150 from the cave and 50 from the surface; Supplementary Material 4), whose measurements were corrected by the fish standard length (FSL). The largest spermatozoan total length were recorded for the new lineage, however, when contrasting between environments, for both lineages cave populations showed a larger spermatozoan total length compared with their closer surface lineage (Table 2 and Figure 3). In the flagellum length and head area (Table 2 and Figure 3B,C), cave populations showed the highest mean values in contrast with their closest surface populations. Therefore, old cave populations showed a mean of total sperm length (from head to tail) of 19.44 µm (3.19 corrected by FSL) and SE = 0.19 µm, while the old surface population showed a mean of 17.61 µm (2.70 corrected by FSL), and SE = 0.40 µm. Similarly, the mean of total sperm length for the new cave populations was 21.26 µm (3.19 corrected by FSL), a SE = 0.36 µm, while for the new surface population the mean value was 20.53 (2.38 corrected by FSL), with an SE = 0.43. The sperm head area was also consistently increase in the cave's populations from both lineages in contrast with surface, with a mean value for the old caves of 5.539 µm, SE = 0.12, while the mean value for the old surface was 3.86 µm, SE = 0.10. For the new caves the mean was 4.69 µm, SE = 0.09, for the new surface was 5.69 µm, SE = 0.16. Finally, the flagellum length was also larger in the caves than for the surface, with a mean for old caves

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Table 2. Mean, maximum values (Max), minimum values (Min), standard deviation (SD) and standard error (SE) of spermatozoan measurements from both lineages in cave and surface populations of *Astyanax mexicanus*

	Spermatozoan total length (STL)	STL/FSL	Flagellum length (FL)	FL/SL	Head area (HA)	HA/SL
Old cave						
Mean	19.447	3.189	16.713	2.741	5.539	0.908
Max	26.100	4.164	24.900	3.952	9.800	1.556
Min	13.200	2.164	11.400	1.869	2.300	0.377
SD	2.432	0.396	2.318	0.369	1.534	0.250
SE	0.199	0.032	0.189	0.030	0.125	0.020
Pachón						
Mean	18.954	3.213	15.931	2.700	6.297	1.067
Max	24.200	4.102	19.500	3.305	7.900	1.339
Min	13.700	2.322	12.300	2.085	4.400	0.746
SD	2.365	0.401	1.756	0.298	0.852	0.144
SE	0.193	0.033	0.143	0.024	0.070	0.012
Pichijumo						
Mean	19.785	3.140	17.111	2.716	6.532	1.037
Max	26.100	4.143	24.900	3.952	9.800	1.556
Min	14.400	2.286	13.100	2.079	4.700	0.746
SD	2.418	0.384	2.387	0.379	1.407	0.223
SE	0.197	0.031	0.195	0.031	0.115	0.018
Tigre						
Mean	19.610	3.215	17.125	2.807	4.132	0.677
Max	25.400	4.164	23.800	3.902	6.400	1.049
Min	13.200	2.164	11.400	1.869	2.300	0.377
SD	2.481	0.407	2.545	0.417	0.940	0.154
SE	0.203	0.033	0.208	0.034	0.077	0.013
Old surface/Rascón						
Mean	17.611	2.709	15.438	2.375	3.861	0.594
Max	23.800	3.662	20.600	3.169	6.300	0.969
Min	13.400	2.062	11.100	1.708	2.700	0.415
SD	2.843	0.437	2.730	0.420	0.751	0.115
SE	0.402	0.062	0.386	0.059	0.106	0.016
New cave						
Mean	21.267	3.197	16.732	2.515	4.693	0.705
Max	28.700	4.864	26.100	4.424	7.800	1.322
Min	13.800	1.840	9.100	1.213	2.800	0.373
SD	3.620	0.677	3.972	0.774	0.942	0.209
SE	0.362	0.068	0.397	0.077	0.094	0.021
Escondido						
Mean	21.267	3.605	18.297	3.101	5.306	0.899
Max	28.700	4.864	26.100	4.424	7.800	1.322
Min	13.800	2.339	12.100	2.051	3.300	0.559
SD	3.638	0.617	3.647	0.618	0.775	0.131
SE	0.364	0.062	0.365	0.062	0.077	0.013

(*Continued*)

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Table 2. (*Continued*)

	Spermatozoan total length (STL)	STL/FSL	Flagellum length (FL)	FL/SL	Head area (HA)	HA/SL
Caballo Moro						
Mean	21.267	2.836	15.301	2.040	4.150	0.553
Max	28.700	3.827	22.800	3.040	6.600	0.880
Min	13.800	1.840	9.100	1.213	2.800	0.373
SD	3.638	0.485	3.772	0.503	0.717	0.096
SE	0.364	0.049	0.377	0.050	0.072	0.010
New surface/Bocatoma River						
Mean	20.536	2.388	17.865	2.077	5.694	0.662
Max	26.500	3.081	22.200	2.581	8.100	0.942
Min	15.500	1.802	13.000	1.512	4.000	0.465
SD	3.040	0.354	2.805	0.326	1.156	0.134
SE	0.430	0.050	0.397	0.046	0.164	0.019

FSL, fish standard length.

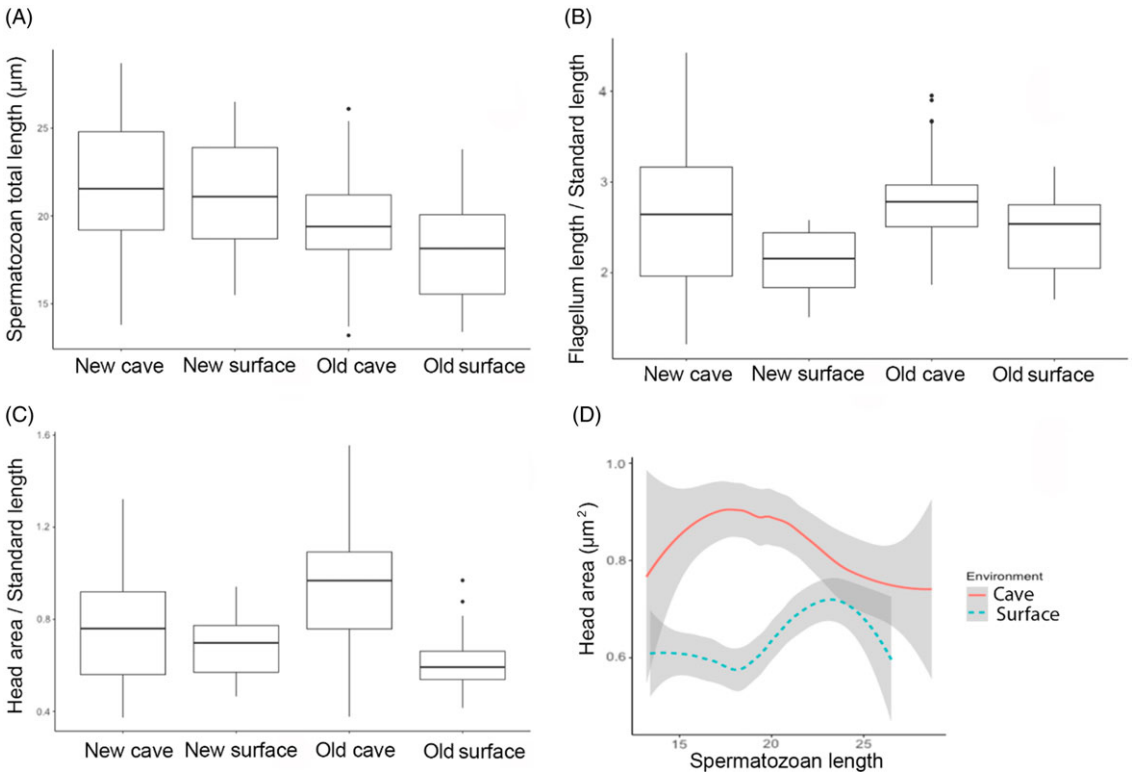

Figure 3. Spermatozoan measurements from both lineages in cave and surface populations of *Astyanax mexicanus*. (A) Spermatozoan total length corrected for the fish standard length by linage and morph combined. (B) Flagellum total length corrected for the fish standard length. (C) Head area corrected for the fish standard. (D) Correlation between head area and spermatozoan total length, grey areas correspond to 95% confidence smoothed adjustment.

of 16.71 µm, SE = 0.09, while for the old surface the mean was 15.43 µm, SE = 0.38, while for the new caves the mean was 16.73, SE = 0.39 while for the new surface the mean was 17.86, SE = 0.397. In the correlation between the head area and the sperm length, we observed that cave populations showed higher head area values than the surface ones to their respective sperm total length (Figure 3D). We found differences in the spermatozoan total length between environments ($F = 9.929$, $P = 0.05$) and linages ($F = 49.86$, $P = 0.005$), however, the interaction between them was not significant. None of the other comparisons were significant.

Oocytes

The oocytes showed spherical conformation with a mean diameter of 822.4 ± 194.1 µm for the surface populations and 604.6 ± 38.3

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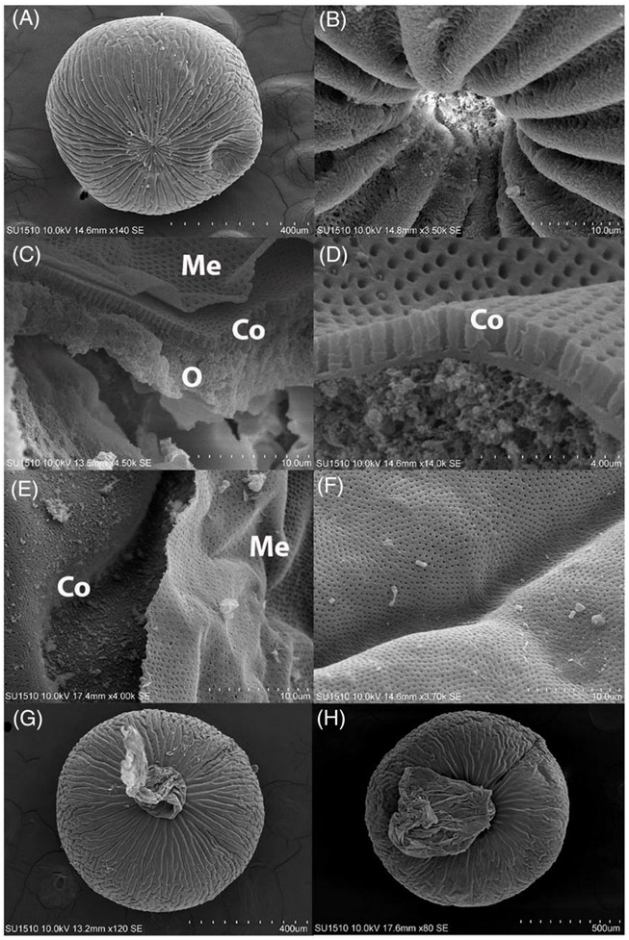

Figure 4. Overview of *Astyanax mexicanus* oocyte. (A) Complete oocyte showing its spherical conformation and the grooves of the oocyte. (B) Micropyle and the grooves, the micropyle was characterized as a small canal in the centre of the grooves. (C) It is possible to observe the three layers of the oocyte: (O) ooplasm, (Co) chorion and (Me) outer membrane. (D) Pore channels present at the chorion. (E) Outer membrane and the chorion are observed, both present pore channels. (F) Porous surface of the chorion. (G) Outer membrane obstruction of the micropyle after fertilization in oocytes from the Pachón cave. (H) Oocyte from the Bocatoma surface population.

μm for the cave populations. It is possible to distinguish the micropyle region between the grooves arranged radially (Figure 4A). The micropyle was characterized as a small channel in the centre of the grooves (Figure 4B). The grooves were perforated by fine pores. We could identify three layers of the oocyte, the outer membrane, followed by the chorion, and in the inner part ooplasm (Figure 4C–F). For those eggs that we recovered fertilized, a micropyle plug was observed, and it was formed by the outer membrane of the chorion, which presented a slight adhesiveness to the aquarium. This plug was present in both surface and cavefish populations (Figure 4G,H).

The general conformation of the oocytes was consistent between caves and surface populations (Figure 5). The oocytes from the Bocatoma surface population were the ones with the largest diameter (mean of 822.4 ± 194.1 μm), in contrast with the other populations including the cavefish populations (mean of 604.6 ± 38.3 μm). Bocatoma surface fish also presented the largest diameter of the micropyle (12.9 ± 0.8 μm) in contrast with the mean observed to the cavefish populations (6.6 ± 1.6 μm). The chorion thickness was larger in the cavefish populations in comparison with the surface (1.3 ± 0.1 μm vs. 1.1 ± 0.2 μm respectively; Table 3).

Discussion

Water quality effect on gametes maturation

The values recorded for water quality during our experiment were close to the optimal conditions previously reported for the species' reproduction (e.g. Hinaux *et al.*, 2011). In this regard, conductivity represents an important parameter whose optimal values for species reproduction have been reported to be between 470 and 590 μs/cm, we observed an increase as time passed, from 439–471.6 μs/cm, with a water salinity from 0.21–0.23 psu. Regarding the pH, the optimum reported was between 8.1 and 8.2, however, in our study we presented lower values of pH between 6.7 and 7.3. For the other parameters measured, we observed values of $NO_2 < 2.8$ mg/l, $NO_3 < 20.6$ mg/l, $NH_4 < 1.9$ mg/l, and $PO_4 < 0.9$ mg/l. The values presented increase the previously reported ranges for some parameters (Hinaux *et al.*, 2011), therefore this information could be relevant for better understanding the range of water conditions for the reproduction of cave and surface populations of *Astyanax mexicanus* in captivity. Physicochemical conditions in the wild could vary widely between the different regions (i.e. El Abra, Guatemala and Micos), and among the caves. In this regard, we registered in the wild a range of pH between 6.7 (Tinaja cave) and 8.1 (Micos), while for conductivity we registered a wide range from 226 (Micos) to 977 (Pachón). Therefore, we suggest that more than a particular value in these parameters, a drastic change, tentatively due to the rainy season, could be triggering the reproduction the wild, but this requires further investigation.

Temperature and hormone considerations for gametes maturation

The three different incubation temperatures used to induce gamete maturation showed reproductive stimulation in 16 of the 26 males used (61.5%) and in two of the seven females used (28.6%). A faster spermiation at higher temperatures has been reported for other species of the genus *Astyanax* (Pinheiro *et al.*, 2020; Postingel Quirino *et al.*, 2021), therefore, we considered that an increase in temperature could affect spermatogenesis and spermatozoa maturation times in *Astyanax mexicanus*. However, the thermal stimuli, an increase of +4°C, was not enough in females to trigger spawning in our study populations, as previously described by Hinaux *et al.* (2011) and Borowsky (2008). Moreover, we observed no difference between the three different thermal stimuli applied (Supplementary Material 1), therefore, based on our observations an abrupt change vs. gradual increase in temperature did not differentially affect the maturation across the males used in the study, however further studies with a larger sample size could allow us to statistically test these observations.

Conversely, the use of hormonal methods began in 1930 with the injection of a crude extract from the pituitary of mature fish [with high levels of gonadotropin hormone (GtH)] to induce spawning (Zohar and Mylonas, 2001). At this time, several synthetic compounds of gonadotropin-releasing hormone (GnRHa) are used, applied from simple injections in physiological serum to microspheres, solving numerous reproductive problems in several species (Zohar, 1988; Van Winkoop *et al.*, 1994; Donaldson *et al.*, 1996; Patiño *et al.*, 2001; Solar, 2002; Munakata and Kobayashi, 2010). In another species of *Astyanax*, *A. altiparanae*, a commercial crude carp pituitary (i.e. Danúbio Aquacultura), has successfully induced males spermiation, under controlled

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Table 3. Mean ± standard deviation of the measurements obtained from the oocytes of cave and surface populations of *Astyanax mexicanus*

Population	Obtained oocytes	Oocyte diameter ($n = 10$)	Micropyle diameter ($n = 5$)	Chorion thickness ($n = 10$)
Sótano de Caballo Moro	60	555.9 ± 145.9	8.5 ± 0.3	1.3 ± 0.1
Sótano de Vázquez	38	638.3 ± 37.8	6.7 ± 1.1	1.3 ± 0.1
Sótano de Escondido	958	586.4 ± 90.1	5.5 ± 1.5	1.1 ± 0.2
Sótano de Tigre	6	589.1 ± 74.9	4.6 ± 0.8	1.5 ± 0.4
Sótano de Pichijumo	484	596.5 ± 63.3	8.7 ± 2.7	1.3 ± 0.1
Pachón cave	450	661.2 ± 50.1	5.9 ± 2.1	1.4 ± 0.2
Bocatoma river	950	822.4 ± 194.1	12.9 ± 0.8	1.1 ± 0.2

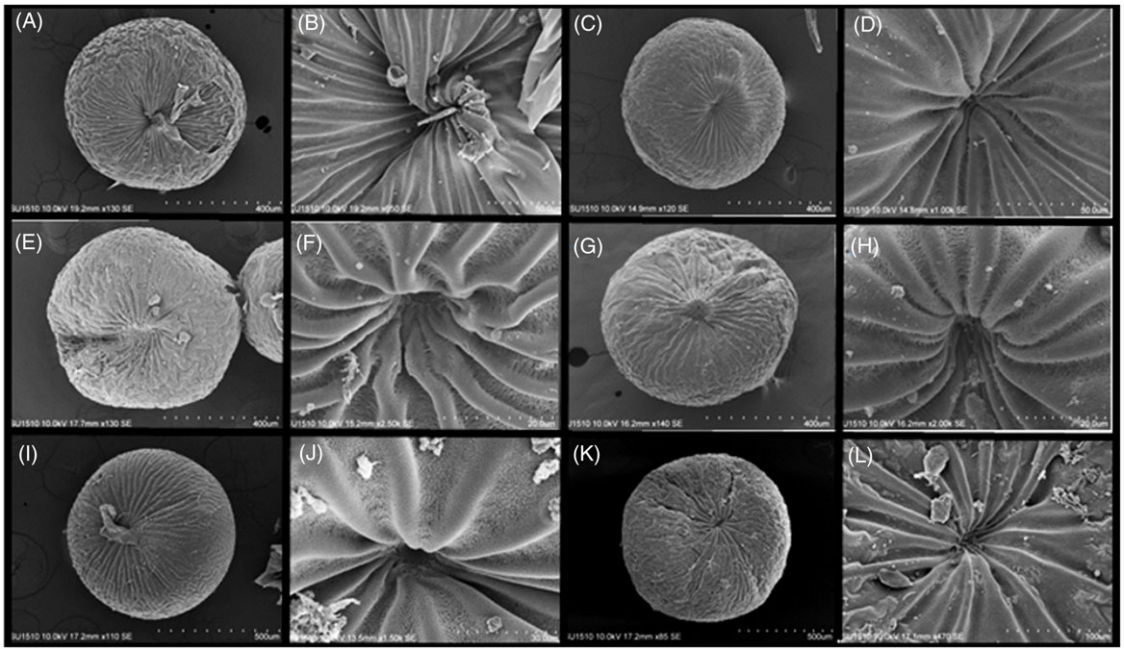

Figure 5. Oocytes and micropyles from the different cave and surface populations of *Astyanax mexicanus*. (A, B) Sótano de Caballo Moro, (C, D) Sótano de Escondido, (E, F) Sótano de Tigre, (G, H) Sótano de Pichijumo, (I, J) Pachón cave, and (K, L) Bocatoma surface population.

temperature conditions, as at higher temperatures the spermiation was faster (see da Silva *et al.*, 2021).

In our study, an analogue of the salmon gonadotropin-releasing hormone (sGnRHa), together with Domperidone (Zohar, 1988; Peter and Yu, 1997) has been proven to induce reproduction in *Astyanax mexicanus*, similar to other species (Peter *et al.*, 1988; Zohar *et al.*, 1995; Patiño, 1997; Peter and Yu, 1997; Zohar and Mylonas, 2001; Munakata and Kobayashi, 2010; Vazirzadeh *et al.*, 2011). Therefore, we observed that Ovaprim, as it is commercially known, is effective in inducing gamete development in both sexes. But as a determinant of final oocyte maturation, ovulation and spawning in *Astyanax mexicanus*, in both troglobian and surface populations (i.e. Pachón, Pichijumo and Escondido caves and the Bocatoma surface population), however, we could not discern the possible combined effect of the hormone together with environmental conditions (i.e. feeding and water quality), as well as temperature and photoperiod (12 h light/12 h darkness). In particular, the temperature, as was previously suggested for other species of the genus (da Silva *et al.*, 2021), could favour sperm maturation and quality, as males exposed for 24 h at 25°C with a neutral pH showed higher sperm concentration in comparison with 20°C or 30°C, and reduced those differences after 96 h of exposure. Further studies evaluating the effect of each of these parameters could shed light on the relative importance of each of them. However, what we can say is that the number of spawned eggs was considerably larger after hormone application, in contrast with not applying the hormone, therefore, the efficacy of the hormone can be verified by contrasting the number of oocytes between hormone vs. thermal stimuli, being over 100 eggs using Ovaprim, and slightly up to 60 with thermal stimuli. With these results, we could confirm that the hormonal combination of sGnRHa + Domperidone (Ovaprim) could be used to induce final oocyte maturation and spawning in *Astyanax mexicanus* species. It could also be used to obtain gametes for interspecific hybridization, and between phenotypes through *in vitro* fertilization, valuable for developmental biology studies and evaluation of trait inheritance.

It is important to contrast our results with those previously published (please refer to Simon *et al.*, 2019). The number of eggs per spawning in our study was considerably lower, that is, previous studies have reported spawning for the surface fish of ~3589 eggs, whereas in our study the largest number of eggs obtained was for the Bocatoma surface population with 950 eggs. For the cave fish, it

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was previously reported, in total, as 1442 eggs per spawning, also a greater number than obtained for our populations of Escondido (958), Pichijumo (484), Pachón (450), Vázquez (38) and Tigre (6). Considering this, some other factors, in addition to those previously proposed (i.e. feeding and water conditions) such as fish age, body condition, etc. could also play a role in the number of spawned eggs (Kjørsvik *et al.*, 1990; Palacios *et al.*, 1995), that could be further investigated.

Maturation stages of Astyanax mexicanus *gametes*

Little information is known about the oocytes and spermatozoa maturation process in *Astyanax mexicanus*. In other species, spermatogenesis duration could be ~6 days for animals kept at 27°C, or 1 day for those kept at 32°C (Postingel Quirino *et al.*, 2021). We obtained mature spermatozoa after 4 days from animals kept at 26°C, for both abrupt or gradual increases (please refer to Supplementary Material 1). The Rascón surface population was the one with the highest spermatozoa viability (99%), followed by Sótano de Tigre and Pichijumo (80% and 75%, respectively). In contrast, Sótano de Vázquez showed the lowest spermatozoa viability (3%), with only two spermatozoa fully mature, tentatively associated with the age of the males. Therefore, in further studies, a deeper consideration of male age could help to elucidate the correlation between spermatogenesis and senescence, together with other environmental factors such as temperature.

Regarding oocyte maturation, most teleosts comprise five different phases in the oocytes, which according to the diameter is divided into early oocytes (10–25 μm), primary growth (25–270 μm), secondary growth (270–380 μm), vitellogenesis (380–650 μm) and maturation (650–1150 μm) (Cárdenas *et al.*, 2008). Based on a previous study, we classified our oocyte maturation stages following Cárdenas *et al.* (2008). Therefore, the oocytes collected from our *Astyanax mexicanus* populations were between the vitellogenesis and maturation phase (555.9–822.4 μm). Kaviani *et al.* (2013) described a similar classification for oocyte maturation stages in zebrafish (*Danio rerio*), so when comparing those with our *Astyanax mexicanus*, our oocytes were at the maturation stage. In zebrafish the diameter of the oocytes in this phase was 412.75 μm and the thickness of the chorion was 1.6 μm, very similar to values observed for our *Astyanax mexicanus* oocytes (555.9–822.4 μm, 1.1–1.5 μm respectively). In contrast with zebrafish a greater oocyte diameter was obtained for cave populations (604.6 ± 38.3 μm), and a considerably lower diameter with respect to the Bocatoma surface oocyte diameter (822.4 ± 194.1 μm).

Morphological features of Astyanax mexicanus *gametes*

Regarding gametes morphology, we observed several similarities with other species of the *Astyanax* genus (i.e. *A. altaparinae*; please refer to Dos Santos *et al.*, 2016), therefore the sperm morphology of *Astyanax mexicanus* was made up of the sperm head, a midpiece, and tail or flagellum. The spermatozoan total length showed significant differences between environments and lineages, suggesting that the new lineage presented a higher spermatozoan total length and, in contrast between environments, caves presented a larger spermatozoan than their closer surface population. The mean observed values for the spermatozoan total length value for old caves was 19.77 μm, SE = 0.19, whereas for the old surface it was 17.611 μm, SE 0.40. The new caves showed a mean value of 21.26 μm, SE = 0.36, whereas for the new surface it was 20.53 μm, SE 0.43. The observed values were similar to those previously reported in *Astyanax altiparanae* (21.22 ± 4.29 μm). The sperm head area was also consistently larger in the cave's populations from both lineages in contrast with surface area, however those differences were not significant. Finally, the flagellum length was also larger in the caves compared with for the surface, with a mean for old caves of 16.71 μm, SE = 0.09, whereas for the old surface the mean was 15.43 μm, SE = 0.38, for the new caves the mean was 16.73, SE = 0.39 and for the new surface the mean was 17.86, SE = 0.397, in contrast with the 18.67 ± 4.32 μm in *Astyanax altiparanae*. It is worth saying that after correcting the flagellum lengths values for FSL, the trend that cave populations were larger than surface was maintained; however, no significant differences were detected between the environments, lineages, or their interactions. The suggestion that *Astyanax mexicanus* cave embryos were larger (Hüppop, 1988), and presented a greater yolk volume than the surface populations (Hüppop and Wilkens, 1991) was supported by the assumption of poorer nutrient conditions in the caves. This is the first study to report larger spermatozoan total length in the caves, which was also accompanied by a larger head area, tentatively suggesting a differential gamete size between cave and surface populations. Further studies controlling some life-related traits such as male age, could help us to better understand the role of spermatozoan size differences between the two contrasting morphs (surface and cave morphs), and to better understand the environmental effect.

The oocytes showed a spherical conformation with a mean diameter of 822.4 ± 194.1 μm for the surface populations, and 604.6 ± 38.3 μm for the cave populations, in contrast with 695.119 ± 3.20 μm in *Astyanax altiparanae* and 1.03 ± 0.03 mm in *Astyanax bimaculatus*. The zona radiata covering the egg is a complex extracellular matrix that after the spawning undergoes extensive molecular modifications that lead to the formation of the chorion (Yamagami *et al.*, 1992; Rizzo *et al.*, 2002). The chorionic structure can show adaptations to the environment, for example it has been reported that the eggs of pelagic fish can have a thinner chorion, whereas demersal fish can have a thicker chorion and a more complex chorionic membrane (Rizzo *et al.*, 2002). In this regard, we observed a slight variation in the thickness of the chorion between the surface and cavefish populations (1.3 ± 0.1 μm vs. 1.1 ± 0.2 μm, respectively; Table 3), however our sample size was relatively low, therefore further analysis including a larger sample size could shed light on the differences between these two contrasting environments in the chorion thickness.

In our study system model, *Astyanax mexicanus*, the oocyte presents at its surface ridges and grooves are arranged radially towards the micropyle, which has been suggested to help to direct the sperm towards the micropyle for fertilization to occur (Rizzo *et al.*, 2002; Dos Santos *et al.*, 2016).

Conversely, several factors contributed to preventing polyspermy in teleost fish (Kobayashi and Yamamoto, 1981; Hart, 1990). One of these was the diameter of the micropyle canal, which is close to the size of the sperm head, in this case we did not measure the micropyle canal, however in future studies longitudinal cuts of the micropyle may give additional information about this barrier. Another factor that prevents polyspermia is the cortical reaction that occurs when the sperm enters the oocyte, producing morphofunctional changes in the chorion. In this case we found that the formation of the outer membrane plug obstructed the micropyle zone; in contrast with *Astyanax altaparinae* in which after fertilization a cone has been reported, and which was described as a rounded protrusion that covers the entire area of the micropyle (Dos Santos *et al.*, 2016).

Finally, appendages associated with the outer layer of the zona radiata could suffer physical–chemical changes that allow the

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attachment to different substrata (Rizzo *et al.*, 2002). In Siluriformes species, there is a micropillar disc that helps the adhesiveness of the oocytes, it is very rare to find this micropillar disc in Characiformes (Rizzo *et al.*, 2002). In *Astyanax mexicanus*, the micropyle plug that is formed by the outer membrane of the chorion showed a slight adhesiveness to the aquarium surface; similarly a weak adhesiveness has been reported in other *Astyanax* genus species (i.e. *A. bimaculatus lacustris*), whose zona radiata ridges around the micropyle have been reported as responsible for substrate adhesiveness. However, in our species *Astyanax mexicanus*, this micropyle plug provides a weak adhesiveness to the substrate, future studies considering histological sections together with specialized stains could help to confirm or rule out adhesiveness in the micropyle plug in *Astyanax mexicanus* oocytes.

In conclusion, this study allowed us to expand the knowledge limits of water quality parameters under which it was possible to reproduce *Astyanax mexicanus* in captivity. Furthermore, we could corroborate the relevance of thermal and hormonal stimuli for triggering gametes maturation, together with a protein-rich diet. In some females, the application of the hormonal stimuli was necessary to spawn and, in this regard, the hormone Ovaprim (GnRHa + Domperidone) resulted in being an effective promoter of ovarian development, maturation end of the oocytes and spawning in *Astyanax*. Regarding morphological gamete features, we found significant differences in the spermatozoan total between linages and environments, the caves in each lineage being the largest. One of the most important discoveries of this study corresponded to the detailed description of the zona radiata of the species, particularly the oocyte chorion, which presented ridges and grooves arranged radially towards the micropyle. Also, after fertilization of the oocyte, it was possible to observe the formation of a plug in the micropyle zone conformed by the outer membrane of the chorion, which provided some weak adhesiveness to the substrate. Finally, differences in the chorion thickness between environments were found that could be a response to contrasting environmental conditions. This study is the first morphological description of *Astyanax mexicanus* gametes, including for the first time a morphological characterization of the Sótanos of Vázquez, Tigre and Escondido, which previously were only known from speleological expeditions. Therefore, this study contributes to our knowledge of phenotypic variation and the possible impact on their adaptation to life in the caves.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199422000223

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Conflicts of interest. The authors declare that they have no conflict of interests.

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