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LOUISE N. PEREZ, BERTHA R. MARILUZ, JAMILY LOREN, AMY LIU, MARCOS P. SOUSA, RODRIGO A. P. MARTINS JOHN S. TAYLOR and PATRICIA N. SCHNEIDER

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The subterranean catfish *Phreatobius cisternarum* provides insights into visual adaptations to the phreatic environment

Louise N. Perez¹a, Bertha R. Mariluz¹a, Jamily Lorena², Amy Liu³, Marcos P. Sousa⁴, Rodrigo A. P. Martins⁵ John S. Taylor³ and Patricia N. Schneider¹⁺

¹Instituto de Ciências Biológicas, Universidade Federal do Pará (UFPA), Belém, Pará, Brazil.
²Instituto Tecnológico Vale, Pará, Brazil
³Department of Biology, University of Victoria, Victoria, BC, Canada.
⁴Coordenação de Zoologia, Museu Paraense Emílio Goeldi (MPEG), Belém, Pará, Brazil.
⁵Programa de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil.

**Running title:** Visual adaptations of the subterranean catfish *Phreatobius cisternarum*

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aAuthors contributed equally

*Correspondence:
Patricia N. Schneider, Centro de Genômica e Biologia de Sistemas. Instituto de Ciências Biológicas, Universidade Federal do Pará, Rua Augusto Corrêa #01, Belém, PA, 66075-900, Brasil. E-mail: patricia.schneider@northwestern.edu, schneider@ufpa.br
Abstract

Vertebrate eyes share the same general organization, though species have evolved morphological and functional adaptations to diverse environments. Cave-adapted animals are characterized by a variety of features including eye reduction, loss of body pigmentation, and enhanced non-visual sensory systems. Species that live in perpetual darkness have also evolved sensory mechanisms that are independent of light stimuli. The subterranean catfish *Phreatobius cisternarum* lives in the Amazonian phreatic zone and displays a diversity of morphological features that are similar to those observed in cavefish and appear to be adaptations to life in the dark. Here we combine histological and transcriptome analyses to characterize sensory adaptations of *P. cisternarum* to the subterranean environment. Histological analysis showed that the vestigial eyes of *P. cisternarum* contain a rudimentary lens. Transcriptome analysis revealed a repertoire of eleven visual and non-visual opsins and the expression of 36 genes involved in lens development and maintenance. In contrast to other cavefish species, such as *Astyanax mexicanus*, *Phreatichthys andruzzii*, *Sinocyclocheilus anophthalmus* and *Sinocyclocheilus microphthalmus*, DASPEI neuromast staining patterns did not show an increase in the number of sensory hair cells. Our work reveals unique adaptations in the visual system of *P. cisternarum* to underground habitats and helps to shed light into troglomorphic attributes of subterranean animals.

Introduction

Hypogean species are characterized by the reduction or loss of eyes and pigments, accompanied by enhancement of other sensory structures (Jeffery, 2009; Mohun *et al.*, 2010; Partha *et al.*, 2017; Protas and Jeffery, 2012). In cave-adapted species, eye degeneration appears to be controlled by at least three different genetic programs. In the blind cavefish *Astyanax mexicanus*, degeneration is triggered by, among other mechanisms, *shh* overexpression and subsequent induction of lens cells apoptosis (Yamamoto *et al.*, 2004). Recent studies have shown that the retinal pigment epithelium (RPE) is critical for eye maintenance (Ma *et al.*, 2020). In the blind cave-dwelling fish *Phreatichthys andruzzii*, eye degeneration is controlled by the
apoptosis of retinal neurons, whereas in the eyeless golden-line fish *Sinocyclocheilus anophthalmus*, transcriptome analysis showed that retinal degeneration is associated with downregulation of transcription factors controlling opsin expression.

Surface fishes can detect moving and vibrating water objects using the mechanosensory organs, the neuromasts. These units are distributed alongside the lateral line and is also observed in the head, where it is called cephalic lateral line (Suppl. Fig 2) (Soares and Niemiller, 2018). Notably, to offset the loss of vision, other sensory functions have been enhanced in cavefish species, including those involving neuromasts, barbels, and taste buds (Meng et al., 2013; Stemmer et al., 2015). The genus *Phreatobius* (Siluriformes) includes three species: *P. sanguijuela* *P. dracunculus* and *P. cisternarum* (Fernandez et al., 2007; Muriel-Cunha and Pinna, 2005; Shibatta et al., 2007; Trajano, 2007). These species occur in the Amazon and Paraguay river basins, however, reports regarding their biology, reproductive cycle, ecology, and physiology remain scarce (Fernandez et al., 2007; Muriel-Cunha and Pinna, 2005; Ohara et al., 2016). *Phreatobius cisternarum* inhabits the superficial phreatic layer, which corresponds to the upper saturated stratum of soil found in hand-dug wells. This species is characterized by small eyes and reduction in both body size pigmentation (Fig. 1 A,B, Suppl. Fig. 1 A,B). Besides the six protruding barbels characteristic of catfishes, *P. cisternarum* has a paddle-shaped caudal region formed by a caudal-fin extended dorsally and ventrally by numerous large procurrent rays, ventrally confluent with the anal fin, a strongly prognathous lower jaw, hypertrophied jaw muscles, and a bright red coloration (Shibatta et al., 2007).

Opsins are a diverse group of G-protein-coupled receptors (GPCRs) broadly categorized as ‘visual’ or ‘non-visual’. The best known opsins are visual opsins found in the rod and cone photoreceptor cells of the retina. These opsins detect light when their ligand, a chromophore called 11-cis retinal, undergoes photoisomerization. Fish are notable in having large visual and non-visual opsin repertoires compared to other vertebrates (Beaudry et al., 2017; Lin et al., 2017; Rennison et al., 2012). For example, the four-eyed fish, *Anableps anableps*, and the distantly related zebrafish (*Danio rerio*), have ten visual opsins (Davies
et al., 2015; Owens et al., 2009). Zebrafish also have 32 non-visual opsins (Davies et al., 2015). It is clear that such a large repertoire is not essential for vision or circadian rhythm regulation, as other animals have much smaller repertoires (Scholtyšek and Kelber, 2017). By surveying the transcriptome of P. cisternarum, we characterized the molecular as well as the morphological response of the visual system to life in perpetual darkness. Furthermore, we used a set of histological analysis, immunofluorescence, and neuromast staining approaches to study adaptations of the sensory system in this subterranean species.

Material and Methods

Specimen collection

Fifteen P. cisternarum adults, ranging from 30 mm to 58 mm, were collected from artificial wells in Benevides (Pará, Brazil) and maintained in dark with daily feeding. In addition to the light protected tanks, the light in the room was controlled to keep the fish in the perpetual dark. During feeding, tank protection was removed and lights were kept off. Three specimens of Poecilia reticulata were obtained in the pet trade and were maintained in individual tanks in a recirculating freshwater system at 24 to 28 °C with aeration at the Genomics and Systems Biology Center. Experiments and animal care were performed following animal care guidelines approved by the Animal Care Committee at the Universidade Federal do Para (protocol no. 037-2015).

Library preparation and Illumina sequencing

One specimen of P. cisternarum was anesthetized in 0.1% tricaine solution (Sigma-Aldrich). Total RNA was extracted from its head using TRizol® (Life Technologies), following manufacturer's protocol. Paired-end 150bp libraries were generated with NEXTSeq Mid Output, according to standard protocol (Illumina) and sequenced on Illumina NEXTSeq 500 platform (NCBI Sequence Read Archive project: PRJNA491408 and run: SRR7878036).

De novo transcriptome assembly and gene annotation

Over 190 million raw sequence reads were obtained. Trimmomatic was used with default parameters to remove adapters (Bolger et al., 2014). De novo
transcriptome assembly was performed using standard parameters in Trinity (Grabherr et al., 2011). The completeness of the assembled transcripts was assessed through the Benchmarking Universal Single-Copy Orthologs tool (BUSCO) (Seppey et al., 2019). Mapping was performed by means of Bowtie (Langmead and Salzberg, 2012) using the constructed transcriptome as a reference. StringTie (Pertea et al., 2015) was used to estimate the abundance of transcripts in Transcripts Per Million (TPM). The identification of lens and RPE genes was performed via tBLASTn search tool, and each contig of interest was searched with manual queries of the consensus sequences from Uniprot (Priyam et al., 2019), and Expasy Translate predicted Open Reading Frames (ORFs) (Gasteiger et al., 2005). BLASTp (NCBI) was performed to confirm protein homology.

Characterization of visual and non-visual opsins

Forty-two zebrafish (Danio rerio) opsins (Davies et al., 2015) were used as query sequences in a tBLASTx survey of the P. cisternarum transcriptome (E-value of 10^{-6}). Danio rerio opsins were also used to survey the transcriptome of another species in the order Siluriformes, the channel catfish (Ictalurus punctatus (accession GCF_001660625.1_IpCoco_1.2). Opsins from P. cisternarum, I. punctatus and the 42 D. rerio opsins were aligned using ClustalW in BioEdit (Hall, 1999; Thompson et al., 1994). Introns in the P. cisternarum opsin transcripts that were obvious in this alignment were removed; precise intron-exon boundaries could be identified by comparing the P. cisternarum sequences to D. rerio orthologs on the NCBI Graphical Sequence Viewer. The alignment was then truncated at the 5' and 3' ends because the degree of sequence divergence in these regions made alignments (even when translated into amino acids sequences) unreliable. Maximum Parsimony (MP) phylogenetic trees were reconstructed using MegaX version 10.1.8 (Stecher et al., 2020) and confidence in the topology was evaluated by Bootstrap re-analyses.

DASPEI staining

The fluorescent dye 2-[4-(di-methylamino)styryl]-N-ethylpyridinium iodide (DASPEI; Invitrogen) was used as a vital dye to stain hair cells within
neuromasts. Three *P. cisternarum* and three *P. reticulata* specimens were incubated in an embryo medium containing 0.005% DASPEI for 15 min, anesthetized in 0.1% tricaine solution (Sigma-Aldrich) for 5 minutes, and rinsed once in fresh embryo medium according to a previously established protocol (Yoshizawa et al., 2010). The specimens were analyzed and photographed with a NIKON-Eclipse 80i fluorescence microscope using the NIS-Elements imaging software.

**Histological analysis**

Twelve specimens of *P. cisternarum* were anesthetized in 0.1% tricaine solution (Sigma-Aldrich). The heads were collected and flash-frozen in Tissue-Tek embedding medium (Sakura). Cryosections (20 µm) were obtained on a Leica CM1850 UV cryostat (Leica Biosystems), captured on Color Frost Plus microscope slides (Thermo Fisher Scientific), fixed in 3% paraformaldehyde, and stored at -80°C for further use. Slides were stained with hematoxylin and eosin following standard protocol (Kiernan, 2008).

**Immunostaining**

Slides were incubated with gamma-crystallin antibody (a gift from Dr. Martins) overnight at 4°C following previously established protocol (Cavalheiro et al., 2014). Immunofluorescence reaction was performed with an anti-rabbit secondary antibody (1:200, Sigma-Aldrich, cat# F6005), incubated for 2 hours at room temperature. Fluorescence nuclear counterstaining was performed with DAPI (Sigma-Aldrich, cat# F6057). Images were captured with a NIKON-Eclipse 80i fluorescence microscope and NIS-Elements imaging software.

**Results and Discussion**

**Opsin repertoire in *P. cisternarum***

The transcriptome assembly process generated 1,350,426 contigs with median length of 329 bp (N50 = 668 bp) (Suppl. Table 1 and 2). Our BLAST survey of this assembly using *D. rerio* query sequences uncovered 11 *P. cisternarum* opsins: three visual opsins (*rh1.1*, *rh1.2*, and *lws*), and eight non-visual opsins (*exorh*, *va1*, *va2*, *parietopsin*, *tmt3a*, *opn4m3*, *peropsin*, and *rgr1*). Twenty-three opsins were identified in *I. punctatus*, and no additional *P.*
cisternarum genes were detected when I. punctatus opsin were employed as query sequences. The entire P. cisternarum rh1.1 gene (a single-exon gene) was contained in one contig. The other 10 P. cisternarum opsin were incomplete; each was represented by one or more contigs that included between 44 bp and 747 bp of coding sequence that could be aligned to all other opsin. Despite this length variation, the P. cisternarum opsin were placed into well-supported clades with I. punctatus orthologs (Fig. 2, Suppl. File 1).

The observation that only rh1.1 was full-length and that introns were present in all P. cisternarum opsin other than rh1.1 and rh1.2 suggests that most transcripts are not functional in the specimen studied. However, it is possible that intron retention (IR) is a form of translation regulation providing the potential to generate opsin proteins rapidly in response to light stimulation by splicing pre-existing transcripts. Another possibility is that opsin IR in P. cisternarum influences eye development by generating opsin mRNAs with premature termination codons (PTCs). Opin loss can lead to retinal degeneration in humans (Silverman et al., 2020), thus nonsense-mediated decay (NMD) of intron-containing P. cisternarum opsin transcripts might play a role in eye degeneration.

P. cisternarum retains a rudimentary lens and shows reduced hair cells

Our histological analysis showed that the small eye of P. cisternarum is composed of an RPE and a rudimentary lens, we did not observe the cornea or sclera and the retina layers were absent in all specimens analyzed in this study. The lack of photoreceptor cells is interesting considering that we found opsin expression in the transcriptome. In addition to the histological analysis of RPE, we found RPE transcripts encoding for proteins such as retinoid isomer hydrolase (RPE65), GTP cyclohydrolase 1-like, L-dopachrome tautomerase, RPE-retinal G protein-coupled receptor-like, among others, in the transcriptome (Fig. 3A, Suppl. Table 3). Immunostaining revealed that among the lens-specific genes identified in our transcriptome, gamma-crystallin, a typical lens protein, was mainly expressed in border of the lens (Fig. 3B). In addition, our transcriptome analysis showed the expression of 36 genes that are critical for lens development and maintenance. This suggests that P. cisternarum retained a rudimentary lens, and that ocular reduction might be due to lower levels of
expression of these genes, however, to test this hypothesis, we would need to perform additional gene expression analysis (Suppl. Table 4) (Alunni et al., 2007; Atukorala and Franz-Odendaal, 2018; Cavalheiro et al., 2017; Hooven et al., 2004; Yamamoto et al., 2004).

Gross et al., have shown previously that a set of genes important for lens maintenance and development is exclusively expressed in A. mexicanus surface population (lost in cave population). Comparative analysis of A. mexicanus natural populations identified 16 genes exclusively expressed in surface, but not cave populations (Gross et al., 2013). Among those, we found 8 genes (Rom1, Gnat1, Pde6, Crygm1, Crygm3, Lhx1b, Arr3a and Atp6ap1) in the transcriptome of P. cisternarum (Table 1, Suppl. Files 2-9). These results suggest that the genetic mechanism of ocular reduction in P. cisternarum may be different from that described for A. mexicanus.

In cavefish, an increase in the number and size of neuromasts is one of the mechanisms used to offset eye degeneration (Soares and Niemiller, 2018; Van Trump et al., 2010). Our results showed the presence of hair cells along the reduced lateral line and present in the head, however, not in abundance, as previously reported for A. mexicanus (Van Trump et al., 2010) (Fig. 3C,D). The lack of supernumerary neuromasts in P. cisternarum could be linked to low water currents in these confined water streams of phreatic environments.

Conclusions

Previous studies in cavefish have uncovered genetic and developmental mechanisms underlying eye degeneration in dark-adapted fish. Here, we present evidence that eye degeneration in P. cisternarum might occur via mechanisms distinct from those described for cavefish species. Histological and molecular analyses in adult fish showed that P. cisternarum maintains eye structures such as RPE and a small lens. Our RNA-seq analyses revealed the visual and non-visual opsin repertoire in P. cisternarum, we identified a set of transcripts implicated in the development and maintenance of the RPE, retina as well as lens. Finally, adaptation to a subterranean environment in P. cisternarum appears to have occurred without concomitant enhancement of sensory neuromasts. In sum, our results provide the groundwork for future
studies aimed at identifying the genetic and developmental underpinnings of eye degeneration in *P. cisternarum* and dark-adapted species in general.

**Ethics**

This study was approved by IBAMA/SISBIO under license number 66015-1 and by the Ethics Committee for Animal Research at the Universidade Federal do Pará (protocol number 037-2015).

**Data accessibility**

Sequence data have been deposited in GenBank with the following BioProject accession number: PRJNA491408 and SRA file: SRR7878036.

**Authors’ contributions**

PNS conceived the ideas and experimental design. LNP, BRM and JL collected the data; LNP, JST, RAPM and PNS analyzed the data; MPS and JT performed the transcriptome assembly; JT and AL performed opsin analysis; PNS, BRM and LNP led the writing of the manuscript. All authors contributed critically to the drafts, gave final approval for publication and agree to be held accountable for the work performed therein.

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Conflict of Interest statement. None declared.

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Fig. 1. The *Phreatobius cisternarum* characterization. *P. cisternarum* adult specimen with miniaturization body, a slender head, and six long thin barbels (A). Map showing collecting site of *P. cisternarum* in Benevides-Para-Brazil (the yellow circle indicates the Benevides city) (B). Scale bar: 0.5 cm.
Fig. 2. Phylogenetic tree showing the visual and non-visual opsins of *Phreatobius cisternarum* (in red), *Danio rerio* and *Ictalurus punctatus*. Maximum parsimony tree showing relationships among *Phreatobius cisternarum*, *Ictalurus punctatus* and *Danio rerio* opsins. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. Sequences were truncated at the 5’ and 3’ ends as these regions could not be aligned for all opsins with confidence. *rh1.2* and *va1* sequences from *P. cisternarum* clustered with orthologs from *I. punctatus*, but for these two genes the truncated alignment (Table S1) contained insufficient data to show their respective relationships to *D. rerio* orthologs. Monophyly of *rh1.2* and *va1* clades was well-supported in analyses of longer subfamily-only alignments (not shown). *Danio rerio* has 42 opsins (Davies et al., 2015) and *I. punctatus* has 23 opsins. All were included in a preliminary analysis but only orthologs of *P. cisternarum* opsins were included in these analysis.
Fig. 3. The *Phreatobius cisternarum* remaining eye structures. Hematoxylin and eosin staining of head sections of *P. cisternarum* showing a retinal pigment epithelium (RPE) and lens (A). Immunofluorescence assay showing gamma crystallin expression on the lens (green) and DNA staining with DAPI (blue) (B). The distribution of superficial neuromasts (yellow dots) on the anterior lateral line (head) (C) and posterior lateral line (trunk) (D) of *P. cisternarum* specimens with DASPEI staining. Cryosections are 20 µm thick. Scale bars: 200 µm (A and B) and 2 mm (C and D).
Table 1. Lens genes expressed in the surface population of *A. mexicanus* and RNASEq from the head of *Phreatobius cisternarum*.

| ID                        | Gene symbol | Gene name                                              | TPM    |
|---------------------------|-------------|--------------------------------------------------------|--------|
| TRINITY_DN133315_c2_g7_j1 | Rom1        | Retinal outer segment membrane protein 1              | 0.11575|
| TRINITY_DN145112_c3_g7_j3 | Gnat1       | Guanine nucleotide-binding protein (G protein), alpha transducing activity polypeptide 1 | 0.30194|
| TRINITY_DN138747_c6_g1_i2 | Pde6        | Phosphodiesterase 6G, cGMP-specific, rod              | 0.24273|
| TRINITY_DN139252_c5_g3_i1 | Crygm1      | Crystallin, gamma M1                                  | 0.13917|
| TRINITY_DN304038_c0_g1_i1 | Crygm3      | Crystallin, gamma M3                                  | 0.06959|
| TRINITY_DN131980_c0_g1_i2 | Lhx1b       | LIM class homeodomain protein                          | 0.21923|
| TRINITY_DN146039_c1_g1_i1 | Arr3a       | Arrestin 3, retinal (X-arrestin), like                | 0.08267|
| TRINITY_DN143563_c2_g1_i9 | Atp6ap1     | ATPase, H+ transporting, lysosomal accessory protein 1 | 1.63144|