UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Conserved Opsin Gene Expression in a cichlid fish (*Andinoacara rivulatus*) Across Seasons in Northwestern Ecuador

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RESUMEN

La plasticidad fenotípica permite a los organismos adaptarse a condiciones ambientales cambiantes y la visión representa un sistema ideal para estudiar las respuestas a entornos de luz variable. Este estudio investigó la presencia de plasticidad de expresión de opsinas en el cíclido, *Andinoacara rivulatus*, del río Cube en el noroeste de Ecuador. Se recolectaron peces durante estaciones secas y de lluvia, y se secuenciaron transcriptomas de la retina para identificar y cuantificar la expresión de genes opsina, que son proteínas de los pigmentos visuales. Los datos ambientales revelaron diferencias ambientales significativas en turbidez, conductividad y potencial de oxidación-reducción entre estaciones. Los análisis filogenéticos identificaron que la visión a color de *A. rivulatus* se basa en la expresión de 5 clases de opsinas: LWS, SWS2B, SWS2A, RH1, RH2Aβ y RH2Aα. Esto es consistente con el repertorio típico de opsinas de cíclidos neotropicales. No se encontraron diferencias significativas en los niveles de expresión de opsinas entre estaciones. Por lo tanto, esto sugiere que *A. rivulatus* no posee plasticidad fenotípica en respuesta a las condiciones de luz cambiantes. Futuros estudios podrían explorar otros aspectos de la adaptación visual, como expresión en etapas de desarrollo o escalas de tiempo más largas para proporcionar más información sobre la capacidad de adaptación de los sistemas visuales de los círculos.

Palabras clave: Plasticidad fenotípica, expresión de opsina, visión de cíclidos, ríos intermitentes y adaptación estacional.

ABSTRACT

Phenotypic plasticity allows organisms to adapt rapidly to oscillating environmental conditions, and vision represents an ideal system to study responses to variable light surrounding habitats. This study investigated the presence of opsin expression plasticity in the cichlid, *Andinoacara rivulatus*, from the Cube River in northwestern Ecuador. Fish were collected during dry and rainy seasons, and retinal transcriptomes were sequenced to identify and quantify the expression of opsin genes, which are visual pigment proteins. Environmental data revealed significant environmental differences in turbidity, conductivity, and redox potential between seasons. Phylogenetic analyses identified that color vision in *A. rivulatus* is based on the expression of 5 opsin classes: LWS, SWS2B, SWS2A, RH1, RH2Aβ and RH2Aα. This is consistent with the typical opsin repertoire of Neotropical cichlids. No significant differences in opsin expression levels were found between seasons. Therefore, this suggests that *A. rivulatus* does not possess phenotypic plasticity in response to changing light conditions. Future studies could explore other aspects of visual adaptation, such as developmental stage expression or longer timescales to provide further insight into the adaptive capacity of cichlid visual systems.

Keywords: Phenotypic plasticity, opsin expression, cichlid vision, intermittent rivers and seasonal adaptation.

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INTRODUCTION

Phenotypic plasticity allows organisms to adapt rapidly to oscillating environmental conditions induced by local seasonality. Vision in animal sensory systems is a great framework to study the impacts of a changing light environment and to evaluate the presence/absence of phenotypic plasticity (Yourick, 2021). In vertebrates, light detection starts with a visual pigment formed by an opsin protein bound to a light-sensitive chromophore (11 cis-retinal) within rod and cone photoreceptors in the retina (Carleton et al., 2020; Hauser & Chang, 2017). When photons hit the light-sensitive photoreceptors, they trigger a chain of molecular interactions, called a transduction cascade. This cascade leads to an electrical signal being sent to the brain through the neurons (Musilova et al., 2021; Nandamuri et al., 2017). Thus, the capacity of an organism to perceive and react to light depends on the absorption of the visual pigment within the cone photoreceptors (Nandamuri et al., 2017; Yourick, 2021). In teleosts, certain subsets of opsins exhibit spectral sensitivities that correspond to the dominant wavelengths in their current habitat (Musilova et al., 2021; Yourick, 2021).

Overall, visual pigments, are involved in the first steps of vision, allowing animals to detect specific light and influence an organism's visual sensitivity and color reception. Vertebrates evolved 5 opsin classes sensitive to different parts of the wavelength spectrum around 500 MYA: a Rod opsin (RH1 - blue-green light), Short wavelength sensitive opsins (SWS1 (UV light) and SWS2 (violet-blue light), Rhodopsin-like 2 opsin (RH2 - green light), Long wavelength sensitive opsin (LWS - red light) (Musilova et al., 2021). Each of these opsins classes have a different maximal absorbance in the wavelength spectrum due to their different amino acid sequences that interact with the ligh-sensitive cromophore in the opsin

pocket, resulting in different visual sensitivities when they form visual pigments in photoreceptors. Other ways in which organism have shown variation in visual sensitivities is achieved by changing the complement of expressed opsin genes (Carleton, 2009). Even though they could have multiple opsins in the genome, some fish for example, express only a subset of three (Carleton & Kocher, 2001). By expressing different opsins sensitive to different parts of the wavelength spectrum, fish attain different visual sensitivities by differential opsin expression.

Fish represent exceptional models for visual studies on gene expression and adaptation. They exhibit one of the highest opsin diversities, possessing up to 40 distinct opsin genes (Musilova et al., 2021). Through gene evolution, fish have had gene duplications and losses, causing certain groups to have different number of sets derived from the 5 original classes (Musilova et al., 2021). African cichlids, as a product of gene duplications throughout their evolutionary history, have 3 genes descended from RH2 (RH2B, RH2Aα and RH2Aβ) and 2 genes from SWS (SWS2A and SWS2B) (Nandamuri et al., 2017). However, Amazonian cichlids primarily have SWS2a, RH2a, and LWS (Carleton, 2009; Hauser & Chang, 2017). This underlies the extensive molecular evolutionary traits and research that remains unclear in opsin gene expression across various light environments (Costa et al., 2013; Musilova et al., 2021).

Research from cichlids in Lake Malawi (*Metriaclima mbenji* and *Metriaclima benetos*) has shown the ability of cichlids to effectively respond to different environments. Although most species expressed long-wavelength-sensitive opsins such as SWS2A, RH2A and LWS suited to turbid waters where they were artificially raised, some fishes also maintained plasticity to express additional short-wavelength opsins SWS1 and SWS2B when exposed to UV light in the tanks, enhancing vision in clear shallows. Such plasticity likely

aided past adaptation to Lake Malawi's fluctuating "blue" and algae-rich "green" periods. These findings suggest adult plasticity builds on developmental programs involving opsins like SWS1 (Nandamuri et al., 2017).

In a study of visual systems across 35 species of Neotropical cichlids, results showed that species expressed the long wavelength opsin of SWS2A, RH2A, and LWS, which is best suited for their turbid habitats. Species of clear water (collected in Lakes) expressed the shorter wavelength opsins SWS2B and RH2B. This suggests spectral tuning of sensitivity across the underwater light gradient and flexibility in opsin expression profiles demonstrates the impressive adaptive precision of the visual system (Torres-Dowdall et al., 2021).

Opsin expression plasticity has been explored in African, North, and Central American fish species, but few studies have been published analyzing the interactions between the river environment and fish visual systems (Costa et al., 2013) in the Neotropics (Härer et al., 2018). Due to high gene and environmental river biodiversity, fish species may reveal complex visual systems and adaptation capacity in the Neotropical taxa (Torres-Dowdall et al., 2017).

Intermittent rivers are rivers and streams that constitute more than 50% of rivers on Earth (Costigan et al., 2016). They play an important role in the variability of water flow, defined by seasonal differences and water supply. They are characterized by prolonged periods of drought and flooding. These seasonal fluctuations fundamentally transform ecosystems and their functions (Datry et al., 2014). Murky water tends to exhibit only transmission of long wavelength light (yellow/red/orange 580-620nm) (Escobar-Camacho et al., 2017), due to the absorption of shorth wavelength by scattering suspended particles. In the dry season, there is less organic material water and tends to be clear, enabling the transmission of shorter wavelength light (blue/UV 380-450nm). Therefore, there is a

significant difference in environmental conditions in both seasons in terms of aquatic habitat, flow velocity, sediment transport and light transmission (Fritz et al., 2017).

Taking into account the vast literature studying fish visual systems and the unique features of intermittent rivers, the main objective of this study is to evaluate the presence of phenotypic plasticity capacity in the vision of the cichlid fish: *Andinoacara rivulatus*. We examined the presence or absence of differential opsin gene expression in *A. rivulatus* communities with different environmental natural conditions in an intermittent system.

We aimed to study the phenotypic plasticity of *A. rivulatus* in intermitted rivers of the Cube River Basin by comparing opsin gene expression in fish collected in different seasons (dry/less turbidity vs wet/more turbidity). Given that the complete opsin set of *A. rivulatus* is unknow and because the overall expression profile of neotropical cichlids is long wavelength shifted, our main hypothesis is that this species exhibits opsin expression plasticity to adapt to changes in the light environment by expressing different opsins in different quantities throughout the wet and dry season.

This study could provide valuable insights on how certain fish species adapt their visual systems to cope with dynamic environmental conditions through phenotypic plasticity using opsin gene expression. By examining differential opsin expression patterns in *Andinoacara rivulatus* between dry and wet seasons, this research aimed to elucidate the molecular mechanisms underlying this remarkable adaptive capacity. Such understanding could not only advance our fundamental knowledge of evolutionary processes but also inform conservation strategies, aquaculture practices, and fisheries management by revealing how organisms perceive and respond to fluctuating visual environments.

METHODS

Study Area and sampling design

Sampling was carried out at the Cube River Basin located in the Choco bioregion, northwestern Ecuador. The Choco bioregion is characterized by having evergreen or lowland foothill forests. Specifically humid forests and cloud forests that receive constant humidity and seasonality, having great wealth of epiphytes, mosses and ferns (Ministerio del Ambiente del Ecuador, 2013; Pujota Pinango, 2020; Salvador Peñaherrera, 2019). Additionally, it contains aquatic ecosystems like rivers, streams and temporary graves that provide a rich habitat for freshwater fish species due to the high levels of endemism and diversity (Aguirre et al., 2021; Benone et al., 2018). The Cube River basin, is characterized by its intermittence and harbors 26 species that survive in this seasonal flooding cycle (Aguirre et al., 2021; Arias et al., 2021; Benone et al., 2018; Camacho et al., 2024; Leberg et al., 2021). It exhibits an altitudinal gradient between 500 and 50 m.a.s.l, combined with multiple land use types in its reaches ranging cacao crops to conserved forests (Aguirre et al., 2021). This makes this river basin an interesting location to study multiple aspects of fish communities and their response to river environmental changes.

Six sampling sites in the Cube River Basin were selected based on habitat representation, access to sampling sites, permission from local communities, and the presence of fish (Table 1). Sampling sites ranged from 1 to 10 meters wide during two distinct hydrological periods in a one-year span, represented by the dry (November 28 - December 1/2022) and flooded season (April 2 - April 7/2023).

During the dry season there some sampling sites consisted of isolated pools whereas in the flooded season these consisted of larger rivers. The objective was to have

representativeness of where species inhabit and contrasting seasonality. Geographic coordinates of the sampling location were recorded using a GPS device for subsequent geospatial mapping (Figure 1).

Table 1. Presents the 6 river sites of FCAT with their geographical information.

Location	Latitude	Longitude	Altitude (m)
Zancudo (Z1)	0.37561	-79.6574	322
Solano (Z2)	0.37817	-79.66441	485
Colorado Claro (Z4)	0.419	-79.64981	194
Colorado Turbio (Z5)	0.41379	-79.64883	206
Cube Naranjal (Z6)	0.52168	-79.64963	76
Estero Naranjal (Z7)	0.51386	-79.64855	80

Study species

Andinoacara rivulatus (Cichlidae: Cichliformes), is a colorful freshwater fish endemic to Ecuador. These fish inhabit both moving and still waters, including rivers, streams, and lakes. They grow up to 30 cm long, with an elongated body shape. Their most distinctive feature is their bright coloration - they have an iridescent green or blue sheen along their flanks, with vertical black bands and red or orange spots on the caudal fin. They breed during October and November; males will care the eggs and aggressively defend their space. This species is poorly studied, in terms of their ecology, behaviors, reproduction, abundance (Jiménez-Prado et al., 2015).

The species *Andinoacara rivulatus* was specifically selected for this study because it is endemic to the region and very common, which makes it easier to catch (Cevallos Chevez, 2020; Jiménez-Prado et al., 2015). Furthermore, there are several research papers that have examined the opsins of African and Central-American cichlids, which provides fundamental information regarding cichlid opsin repertoires and expression (Carleton, 2009; Carleton et

al., 2010; Carleton & Kocher, 2001, 2001; Escobar-Camacho et al., 2017; Härer et al., 2018, 2018; Ricci et al., 2023; Torres-Dowdall et al., 2017, 2021). Thus, this makes *A. rivulatus* an excellent candidate to test hypotheses about differential gene expression and phenotypic plasticity.

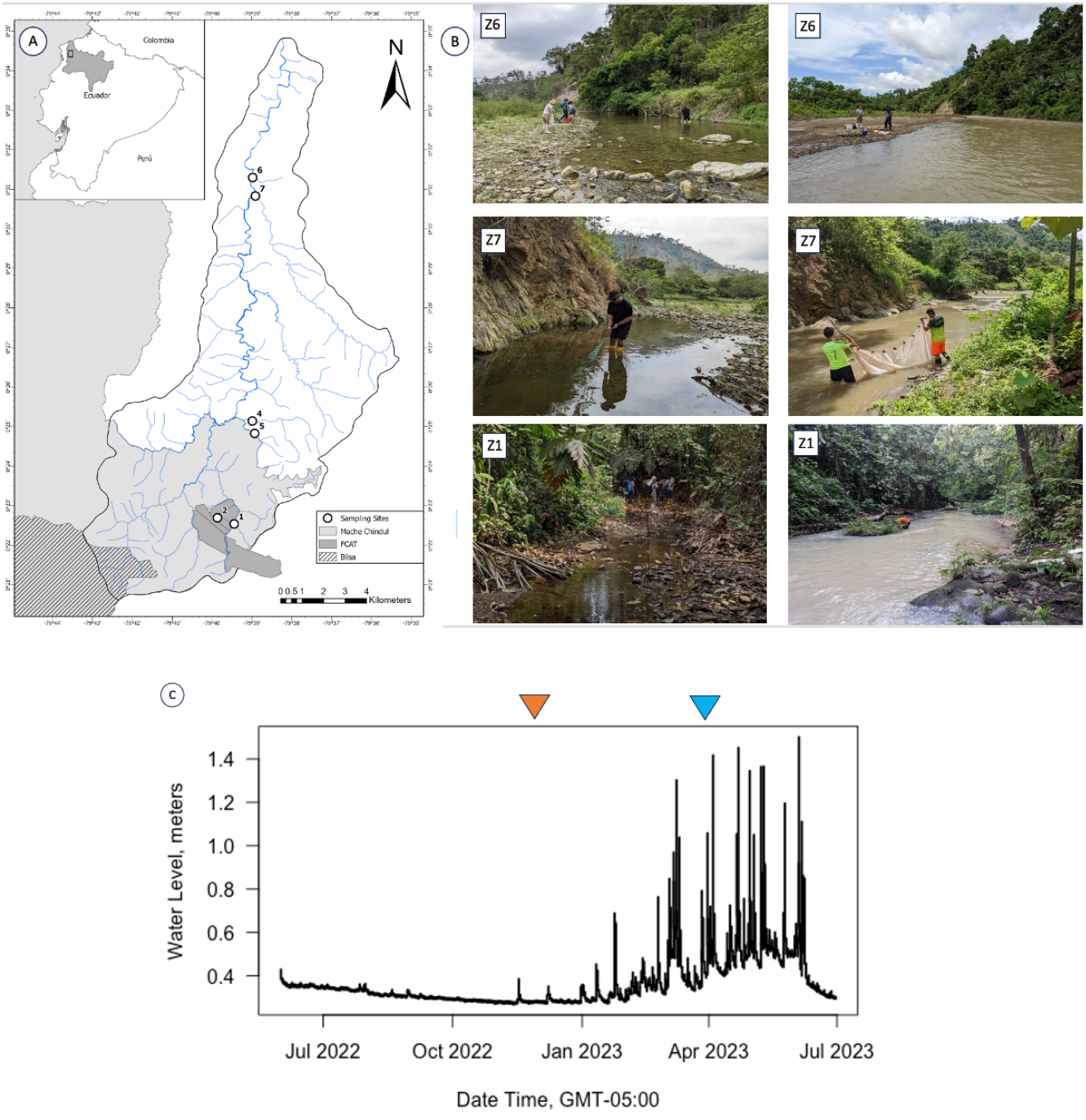

Figure 1. Study area and water level (m) fluctuation of the Cube River Basin

A) Geographical visualization of sampling sites in the Cube River basin. B) Sampling sites Z6, Z7, and Z1 during the dry (left) and flooded (right) season. C) The relationship between water level (meters) and the date time in one year collected from loggers at Z1. The orange triangle represents the dry season, while the blue triangle is the flooded season. Hourly streamflow at reach 7 between 28/11/2022 – 01/11/2022 and 02/04/2023 – 07/04/2023 to highlight the strong degree of hydrological intermittency in the headwater of the study area. Water level data was recorded every 15-minutes using pressure transducer (Hobo U-20). Periodic streamflow measurements were taken using the velocimeter-area method electromagnetic flow meter (MF, Pro, (MF Pro, OTT®) and were used to convert water level into a continuous streamflow time series.

Environmental variables

At each location during sampling, several physicochemical variables were measured using a YSI multi-parameter water quality sonde, these variables included temperature (°F), turbidity (FNU), dissolved oxygen (mg/L and % saturation), conductivity (µS/cm), pH, oxidation-reduction potential (ORP). Light penetration at sampling sites was assessed with a Secchi disk. For this, two Secchi disks measurements were performed in random pools at sampling sites and in two different depths. On the next sampling season, these measurements were repeated at different pools within the same sampling site at the same depth as the first sampling season.

In order to analyze the significance and difference of environmental variables in a t-test was conducted in R studio (Rstudio, Boston, MA, USA). Representation of data collected and data distribution in the two seasons was visualized with boxplots and linear graphs.

Fish sampling

To collect fish we used hand nets, stop nets, and seine nets with a 5-millimeter mesh size, lengths of either 6 or 10 meters, and a height of 2 meters. Sampling was performed by walking along zig-zag transects across the streams at a constant pace, with the ends of each transect blocked using seine nets (Camacho et al., 2024). Each sampling transects consisted of approximately 200 meters. All fish caught during sampling were temporarily held in

buckets with oxygen on site. From these, we selected a few individuals for collection and tissue extraction based on their size (adult) and rarity of the species. Fish were transported to FCAT research station, where they were photographed and anesthetized using eugenol oil (Camacho et al., 2024).

Tissue preparation

At FCAT laboratory all individuals were photographed and then humanely euthanized following an approved chemical method in where they were submerged 10 minutes in a bath of benzocaine (250 mg/liter). Immediately following euthanasia, eyes were carefully dissected from each specimen using microdissection instruments to avoid tissue damage. Intact retinas, including the retinal pigment epithelium, were carefully isolated and immediately transferred into RNAlater solution (ThermoFisher Scientific) for stabilization and preservation of RNA integrity.

Morphological measurements, including total length (TL), fork length (FL), and body weight were recorded prior to preservation (APPENDIX A). Muscle tissue samples were immediately preserved in 96% ethanol for subsequent DNA extraction. Finally, whole fish specimens were fixed in a 10% formaldehyde solution and later stored as vouchers in 70% ethanol following standard museum collection protocols for voucher specimens.

This research was conducted in compliance with animal welfare laws, guidelines, and policies approved by the Ministry of Environment, Water and Ecological Transition (Ministerio de Ambiente, Agua y Transición Ecológica del Ecuador—MAATE). Scientific/Collection research permit (MAATE-ARSFC-2022-2286), Mobilization permit (01038; 01159), Export permit (071-2023-EXP-CM-FAU-DBI/MAATE) and permit to access genetic resources (MAATE-DBI-CM-2023-0289).

RNA extraction

For sequencing, fish retinas were used proportionally across the two seasons, with four individuals from the dry season and five from the flooded season for *A. rivulatus*. Although this species was collected in various site locations, individuals for molecular sequencing were used only from Zancudo (Z1). Total RNA was extracted from retinal tissue samples preserved in RNAlater solution at Fabio Cortesi's lab at Marine Sensory Ecology Group in the School of the Environment and Queensland Brain Institute (© The University of Queensland). The tissue was first digested with Proteinase K enzyme (New England Biolabs) to facilitate RNA release. The Monarch Total RNA Miniprep Kit (NEB) was then utilized to isolate total RNA, followed by treatment with RNase-free DNase (NEB) to remove any contaminating genomic DNA. RNA quality and yield were assessed using the eukaryotic total RNA 6000 Nano assay on a Bioanalyzer 2100 instrument (Agilent Technologies) at the Queensland Brain Institute's facilities (Escobar-Camacho et al., 2017; Fogg et al., 2023).

Retinal transcriptome libraries were prepared from the total RNA samples using the NEBNext Ultra RNA library preparation kit for Illumina (NEB) following the manufacturer's instructions. Library concentrations were quantified using the Qubit dsDNA BR Assay kit (ThermoFisher Scientific) prior to barcoding. The barcoded libraries were pooled in equimolar ratios and subjected to 150 base pair paired-end sequencing on the NovaSeq X Plus (Illumina) using SBS v4 sequencing chemistry (Fogg et al., 2023). Samples were sequenced at Novo gene in Singapore.

Transcriptome assembly with Galaxy

Raw sequencing reads were processed and assembled into transcriptomes using the Galaxy (https://usegalaxy.org) web-based platform. First, quality assessment was performed on the raw forward and reverse FASTQ files using FastQC tool (Galaxy Version 0.74+galaxy0).

Second, quality trimming and adapter removal were then carried out using the TrimGalore tool (Galaxy Version 0.36.6) with the following parameters: paired-end mode with separate input files for forward (R1) and reverse (R2) reads, Illumina TruSeq3 adapter sequence specified for clipping, head crop of 10 bases from read starts, trailing base removal with minimum quality score 20, sliding window trimming (window size 4, average quality 20), and minimum read length 80 bases post-trimming. A second round of FastQC was run on the trimmed paired-end reads to verify quality metrics (Fogg et al., 2023; The Galaxy Community et al., 2022). Third, the trimmed FASTQ files were converted to FASTA format using the FASTQ Groomer tool (Galaxy Version 1.1.5). De novo transcriptome assembly was then performed on the paired FASTA files using the Trinity assembler (Galaxy Version 2.15.+galaxy1) with default parameters (Fogg et al., 2023; The Galaxy Community et al., 2022)

Opsin identification and Phylogenetic Analysis

The assembled transcriptome contigs and unassembled reads files were downloaded from Galaxy for further analysis in Geneious ® (Version 11.0.5) (https://www.geneious.com). To identify opsin gene sequences, the assembled transcriptome was used as the database for tBLASTn searches, queried against cichlid reference opsin sequences from NCBI of species *Amphilophus citrinellus, Amphilophus astorquii* and *Astronotuss ocellatus* of the opsins: LWS, RH1, RH2A, RH2B , SWS2A, and SWS2B.

The BLAST hit sequences of *A. rivulatus* trancriptomes were extracted and combined with reference opsin sequences from related fish species including *Matriaclima zebra* (Cichlidae: Cichliformes), *Danio rerio* (Cyprinidae:Cypriniformes), *Cichla monoculus* (Cichlidae: Cichliformes), *Lucania goodei* (Fundulidae: Cyprinodontiformes), *Amphilophus citrinellus* (Cichlidae: Cichliformes), *Amphilophus astorquii* (Cichlidae: Cichliformes), and

Oryzias latipes (Adrianichthyidae: Beloniformes). Multiple sequence alignments were generated using the MUSCLE algorithm (v3.8.425) within the Geneious Alignment tool.

For the phylogenetic analysis, I utilized the W-IQ-TREE web interface. This tool performed model selection to identify the best evolutionary model, as well as a search to find the optimal phylogenetic tree topology coupled with node maximum likelihood support. The best fit model was LG+F+G4 and support values for the tree were computed using 1000 ultrafast bootstrap replicates and the SH-like aLRT method (Camacho et al., 2024; Trifinopoulos et al., 2016).

With the IQ-Tree file throughout Figtree and Illustrator I made the analysis aimed to determine identify of *A, rivulatus* opsin classes within the phylogenetic tree and their clustering relative to reference sequences obtained from GenBank, allowing for opsin classification (Escobar-Camacho et al., 2024; Trifinopoulos et al., 2016).

Gene expression analysis

In Geneious, we set up paired reads from forward and reverse FASTA files of each sample. We then used our extracted opsin sequences and mapped the paired read files of each sample against each opsin query using the "Map to reference" tool. We set up a sensitivity for mapped reads with a "Maximum Mismatches Per Read" of 2%, to avoid mapping similar opsins. We mapped reads for each opsin of all samples.

To normalize gene expression according to gene sequence length and total number of transcriptomes reads, we calculated expression using Fragments Per Kilobase Million Mapped Fragments (FPKM) of each of 9 individuals on every opsin. FPKM is for paired end RNA-seq data, which means both ends of a fragment can be mapped; giving two reads per fragment or depending on the quality only one end of the paired end (Maza et al., 2013). This helps to keep track of the fragments and makes sure that a fragment is not counted twice; A

higher FPKM means a higher gene expression (Weirick et al., 2016). Generally, it calculates the number of fragments matched to the same transcript (Zhao et al., 2021).

$$\boldsymbol{FPKM} = \frac{ExonMappedFragments * 10^9}{TotalMappedFragments * ExonLength}$$

Equation 1. FPKM calculation (Zhao et al., 2021). Exon represents the opsin sequence.

The FPKM values were obtained for each fish and for each opsin class. Opsin gene expression was then compared between individuals of different seasons with using t-test, Wilcoxon test and Kruskal-Wallis test. All analyses were performed in R studio (Rstudio, Boston, MA, USA).

RESULTS

Seasonal environmental variation

The environmental parameters were compared between the dry and flooded seasons using the R-studio t-test. The results showed no significant differences in temperature ($t = -0.491$, $df = 10$, $p = 0.634$), dissolved oxygen ($t = 0.211$, $df = 10$, $p = 0.837$), and pH ($t = -0.988$, $df = 10$, $p = 0.347$) between the two seasons (Figure 2A). However, there were significant differences in conductivity ($t = 2.82$, $df = 10$, $p = 0.0181$), oxidation-reduction potential (ORP) ($t = -3.64$, $df = 10$, $p = 0.00452$), and turbidity ($t = -3.08$, $df = 9$, $p = 0.0132$). For turbidity, we obtained this result after removing one outlier from the locality known as Solano/Z2 (Table 1), 107.8 NTU, which was unusually high. But including all, we didn't observe significant differences ($t = 0.506$, $df=10$, $p = 0.624$).

Specifically, the conductivity was higher during the dry season compared to the flooded season, indicating a higher concentration of dissolved ions in the water during the dry period. The ORP was significantly lower during the flooding season, suggesting a more reducing environment during this period. Turbidity, as expected, was significantly higher during the flooding season, likely due to the increased suspension of particulate matter in the water column caused by the higher water flow and sediment transport. For the Secchi measurements, the flooded season suggests higher turbidity across depths, in comparison to the dry season which has a lower turbidity but with the same or lower depth. In three data points the Secchi disks were completely lost and had no visibility during the flooded season (Figure 2B).

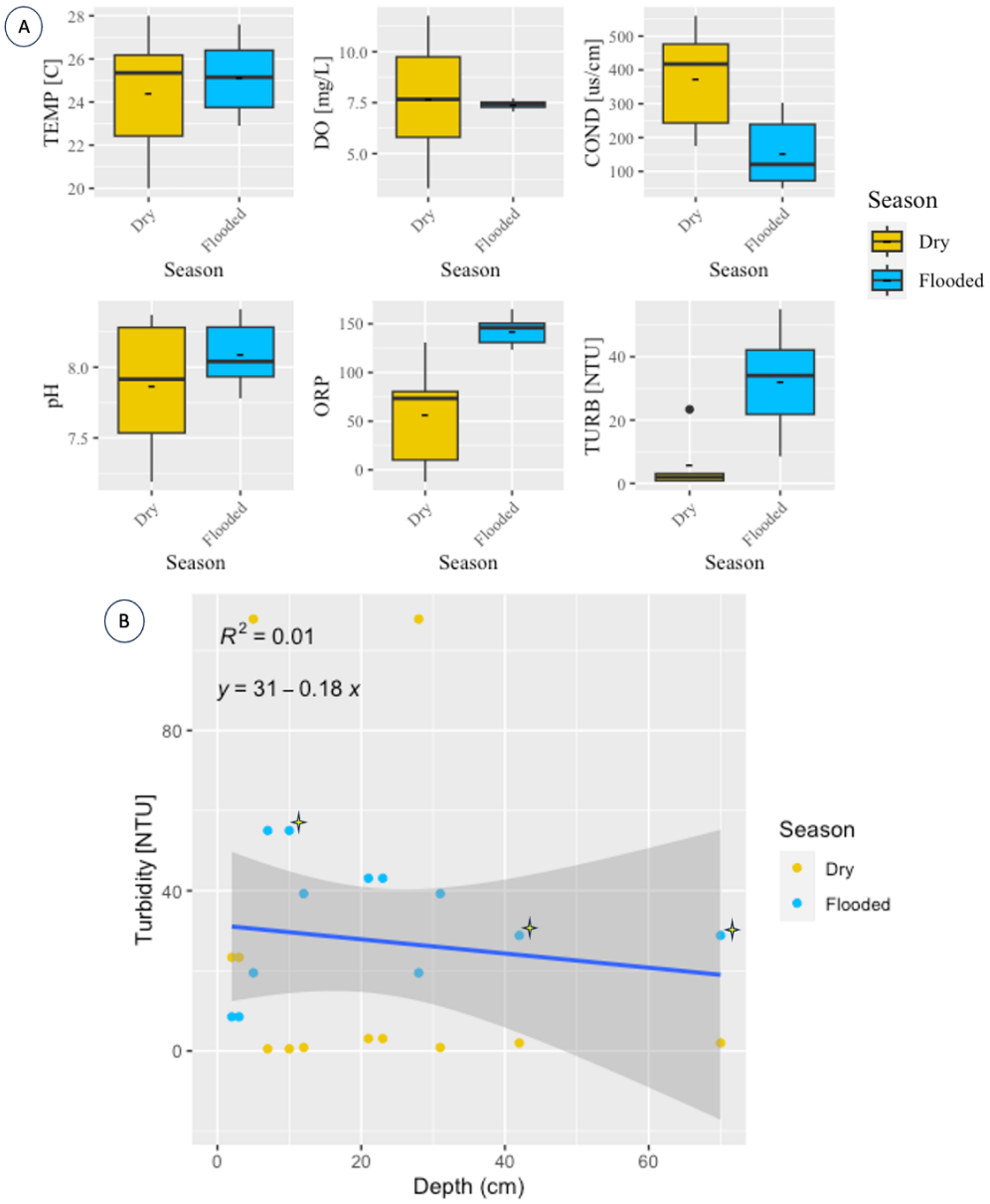

Figure 2. Comparison of Environmental variations in the two seasons studied.

A) Boxplots of conductivity, oxidation-reduction potential, and turbidity present significant differences between dry and flooded season. Temperature, dissolved oxygen, and pH shows no significant differences. B) Relationship between turbidity and depth of water taken by a Secchi disk. Symbol represents values in where the Secchi disk was completely lost and had no visibility in the water. R-squares has a value of 0.01 and the linear function is 31-0.18x.

Opsin repertoire of *Andinoacara rivulatus*

Results of the raw assembly on Trinity in Galaxy (Table 2) showed that the number of sequences assembled between 90,000 to 100,000 bp which presents a high coverage and

sequencing depth. The length of the sequences indicates a high variation in genetic structure, and a Guanine-Cytosine content of around 45-47% provides a good function of the sequences. The total number of reads indicates the number of mapped reads that correspond to each particular transcriptome assembly.

Table 2. Transcriptome information resulted from assembled sequences in Galaxy. Shows for each individual the number of reads, maximum length, minimum length, G-C content and the total number of reads per transcriptome.

Individual	**# Sequences assembled**	**Max Sequence**	**Min Sequence**	**%GC**	**# of reads**
A14	87,520	15,158	191	46.0	19,536,410
A15	96,163	11,367	192	46.0	20,687,119
A16	89,742	10,423	186	45.9	20,874,373
A17	101,652	11,440	187	46.1	20,349,692
A86	84,036	13,398	201	45.6	20,552,687
A87	89,594	15,379	196	46.0	19,536,410
A88	92,128	13.849	184	45.7	23,448,110
A90	94,707	16,747	191	45.8	20,784,061
A92	93,488	11,355	194	45.9	20,682,245

Based on nine transcriptomes (Figure 3) and phylogenetic analyses, our results suggest that *Andinoacara rivulatus* expresses five opsins: LWS, SWS2B, SWS2A, RH1, and RH2A (both duplicates RH2Aβ and RH2Aα). This was evident because opsin sequences of *A. rivulatus* clustered in the respective clades of each opsin class. No opsin sequences were detected for the opsins of SWS1 and RH2B in the transcriptomes.

These opsins found in the transcriptome suggest that *A. rivulatus* color vision is based on at least three different photoreceptor types that may have pure or mixed combinations of

visual pigments. The opsins expressed by *A. rivulatus* constitute visual pigments sensitive to 560nm (LWS), 425nm (SWS2B), 456nm (SWS2A), 447–525 nm (RH1), 517nm (RH2Aβ), and 528nm (RH2Aα) (Musilova et al., 2021; Ricci et al., 2023). RH1 (rhodopsin) does not mediate color vision as it is used for vision in dimlight however is important to note that it was also found expressed across all samples (Figure 3).

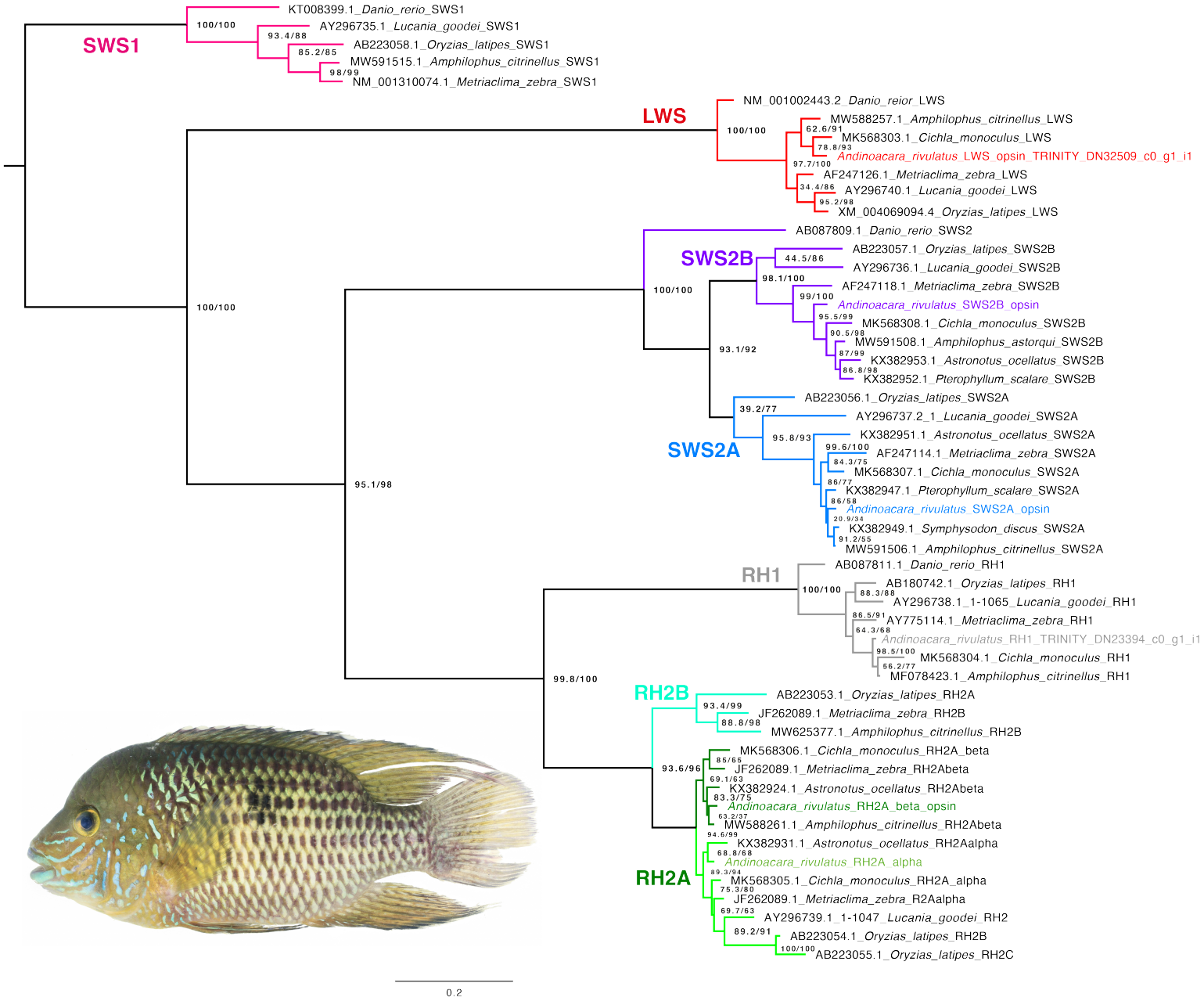

Figure 3. Phylogenetic relationships between opsin gene expressed in *Andinoacara rivulatus* and relative species.

Opsin maximum likelihood phylogenetic tree of *Andinoacara rivulatus, Danio rerio, Lucania goodei, Oryzias latipes, Amphilophus citrinellus, Metriaclima zebra, Cichla monoculus, Amphilophus astorqui, Astronotus ocellatus, Pterophyllum scalare. and Symphysodon discus*. Color shades indicate neotropical *A. rivulatus* opsins in each opsin class. Node statistical support is shown as: SH-aLRT support (%) / ultrafast bootstrap support (%) (Escobar-Camacho et al., 2024).

Opsin gene expression with seasonality

Differential opsin expression analyses showed non-significant differences of opsin expression between seasons for t-test (Table 3), Kruskal-Wallis test (Table 4) and Wilcoxon test (Table 5). Also, boxplot results further supported the absence of significant findings across the various brain regions and sleep stages examined (Figure 4) .

Table 3. t-test results representing the significance of each gene expression level.

Opsin	Df	t value	p-value
SWS2B	7	1.51	0.176
SWS2A	7	0.196	0.3252
RH2Aβ	7	0.784	0.459
RH2Aα	7	0.624	0.553
LWS	7	-0.704	0.504
RH1	7	-0.627	0.551

Source: R Studio

Table 4. Kruskal-Wallis test results representing the significance of each gene expression level.

Opsin	Df	H value	p-value
SWS2B	1	1.23	0.268
SWS2A	1	0	1
RH2Aβ	1	0.960	0.327
RH2Aα	1	0.540	0.462
LWS	1	0.240	0.624
RH1	1	0.0600	0.806

Source: R Studio

Table 5. Wilcoxon test results representing the significance of each gene expression level.

Opsin	W value	p-value
SWS2B	14.5	0.3252
SWS2A	10	1
RH2Aβ	14	0.4127
RH2Aα	13	0.5556
LWS	8	0.7302
RH1	9	0.9048

Source: R Studio

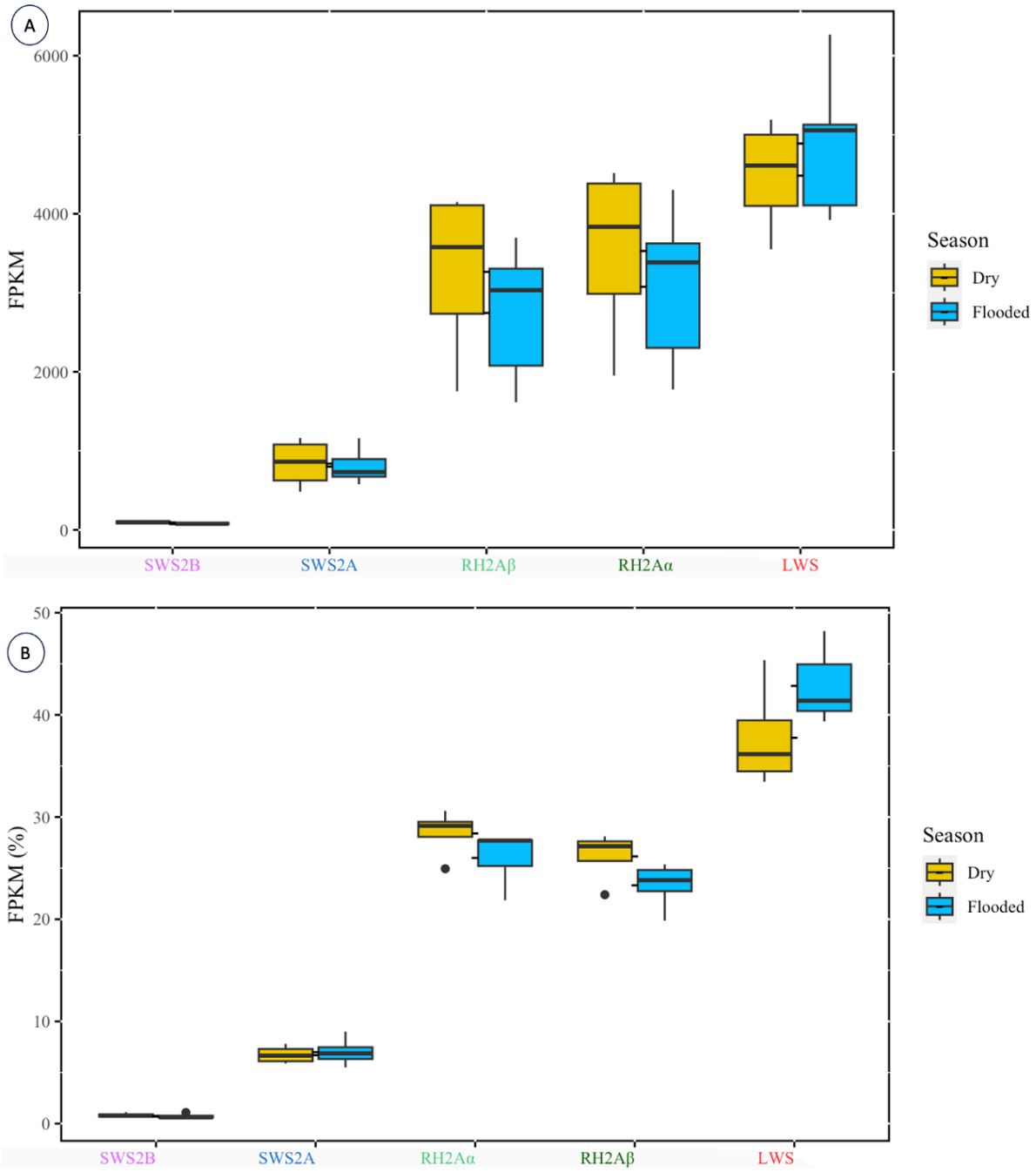

Figure 4. Relative cone opsin expression of *A. rivulatus* based on FPKM values (fragment per kilobase of transcript per million reads).

A) Opsin level expression in FPKM of SWS2B, SWSA, RH2A_beta, RH2A_alpha and LWS during the flooded and dry season. B) Opsin Expression based of FPKM percentage values depending on seasonality.

Aditionally, FPKM results were a great indicator to highligh that *A.rivulatus* presents a higher expression of the long waveleght opsin LWS (mean = 4715.46) in t double cones vision, while SWS2A (mean = 822.86) was dominant in single cones (

Figure *5*).

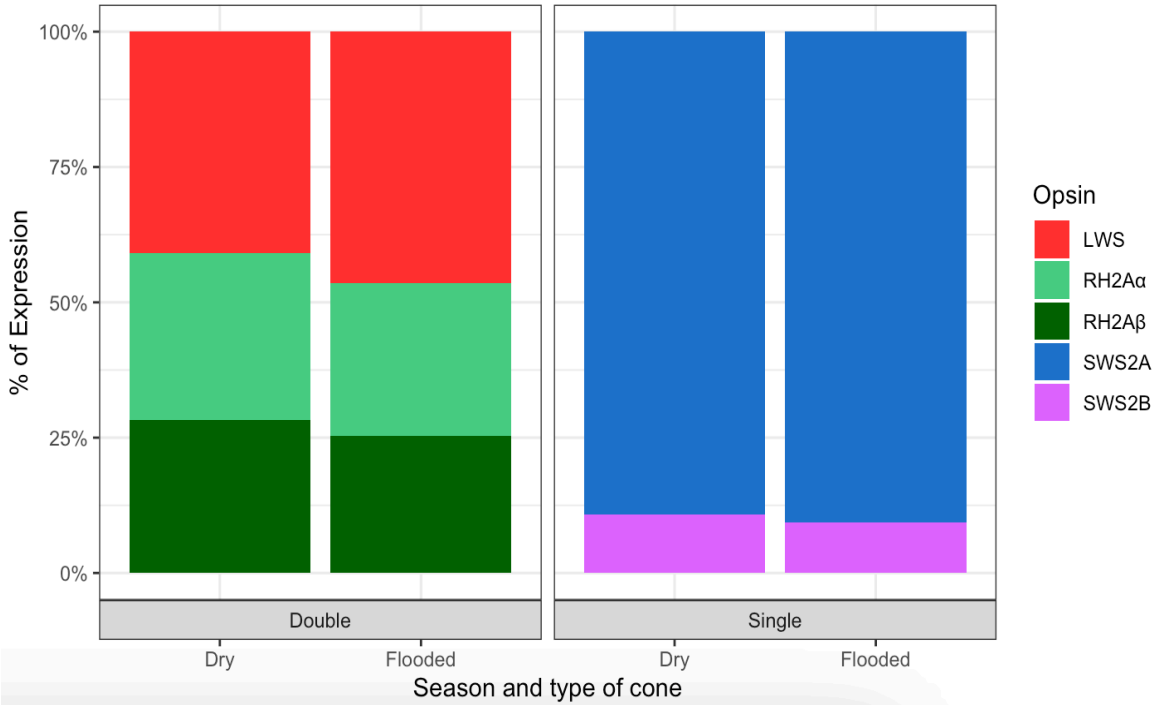

Figure 5. Single and double cone opsin expression (FPKM %) of *A. rivulatus* in relation to seasonality.

DISCUSSION

Seasonal variation in the Cube River basin

The environmental condictiones showed significant differences in turbidity, conductivity, and oxidation-reduction potential (ORP) between the dry and flooded seasons in the Cube River Basin. Turbidity was significantly higher during the flood season, and this is attributed to an increased flow and greater presence of suspended sediments, substrates,

and leaf litter decomposition compared to the rainy period (Machado et al., 2018; Pujota Pinango, 2020). Conductivity increased during the dry season due to evaporative water loss, which concentrated the dissolved ions and increased salinity as the amount of dissolved solids rose, despite initially having few dissolved solid (Molinero et al., 2019; Pujota Pinango, 2020). The lower ORP in the flooded season indicates more reducing conditions, potentially from organic matter inputs and decreased oxygen. As expected, due to the high seasonality in the Choco Andino, these values attribute to the variability in aquatic habitats. Therefore, setting a differentiation within environmental water conditions in intermittent rivers (Arias et al., 2021; Montoya et al., 2006).

In contrast, the parameters of temperature, dissolved oxygen, and pH, did not exhibit significant differences between both seasons. Water temperature can be influenced by solar exposure and atmospheric heating in the dry season, resulting in higher temperatures, while the contribution of colder waters during rainfall events could decrease the temperature during the flooded seasons . Dissolved oxygen levels were expected to be higher during the flooded season due to increased turbulence and water movement. Likewise, pH is usually regulated by natural buffer systems that cushion drastic changes; therefore, it was expected to maintain a pH around 6 (Machado et al., 2018). The lack of significant differences between seasons could be attributed to the fact that these systems are characterized by "flash floods" (Datry et al., 2014; Salvador Peñaherrera, 2019) which means that during the dry season, occasional rainfall events may occur. As shown in (Figure 1C), depicting the hydrogram before data collection, there were rainy days that could have affected the environmental data during the designated dry season.

Opsin repertoire in cichlids

Andinoacara rivulatus presents an opsins repertoire of five opsin genes, matching the sets of other neotropical cichlids (*Pterophyllum scalare, Symphysodon discus, Astronotus ocellatus, Amphilophus citrinellus, Amphilophus labiatus, Amphilophus xiloensis, Amphilophus astorquii, Amphilophus zaliosus, Amphilophus sagittae*) (Escobar-Camacho et al., 2017; Torres-Dowdall et al., 2021). It has been previously studied that African cichlids, like *Oreochromis niloticus* (tilapia of rivers), present seven ancestral cone opsins (SWS1, SWS2B, SWS2A, RH2B, RH2Aβ, RH2Aα, and LWS), while neotropical cichlids exhibit a reduced set of 5 or 6 opsins (Carleton & Kocher, 2001; Nandamuri et al., 2017). Research suggest inactivation or loss of these opsins seems to depend on the environmental conditions in which these species live (Ricci et al., 2023). Therefore, *A. rivulatus* maintains a typical opsin set among Neotropical cichlids. It has been suggested these opsins constitute the necessary visual pigments for the light environment of Neotropical aquatic ecosystems, with greater amount of suspended inorganic particles, organic matter, algae, and sediments, which absorb the short-wavelength light and allow only the passage of long-wavelength photons (Escobar-Camacho et al., 2020; Härer et al., 2018; Torres-Dowdall et al., 2017).

Opsin gene expression profile of *A. rivulatus*

Andinoacara rivulatus exhibited greater expression of long-wavelength opsins (RH2Aβ, RH2Aα, and LWS) than short-wavelength opsins (SW2A, SWS2B) (

Figure *5*). This is typical of cichlid expression because of the cichlid retinal composition where cones are arranged in a mosaic pattern of a single cone surrounded by four double cones (Musilova et al., 2021). Single cones harbor short-wavelength opsins like SWS2A and SWS2B, while double cones are composed of long-wavelength opsins, such as RH2Aβ, RH2Aα, and LWS (Gray, 2021; Torres-Dowdall et al., 2017; Matsuo et al., 2022)).

Thus, *A. rivulatus* higher proportion of long-wavelength opsins coincides with the double- to single-cone ratio 4:1 of cichlids (Torres-Dowdall et al., 2017).

Opsin gene expression between seasons

There were no significant differences in opsin gene expression levels between retinal transcriptome of the dry and flooded seasons. This is surprising because the literature has suggested that some African Cichlids species and Neotropical can exhibit phenotypic plasticity in opsin expression (Carleton, 2009; Härer et al., 2018; Nandamuri et al., 2017). Differential opsin expression has been suggested to occur so that different visual systems of fishes match with their visual sensitivities the available spectrum in the respective light environments (Torres-Dowdall et al., 2021). *Andinoacara rivulatus* may have a more limited capacity for such plasticity. Otherwise, the species might have evolved a relatively fixed opsin expression profile that is maintained during both season and is specific to the ecological needs in light environment. It should be noted that lack of plasticity in opsin expression is not an anomaly, as certain cichlid species (*Aequidens pulcher*) may have evolved a constitutive opsin expression profile tailored to their specific ecological light environment (Carleton et al., 2020; Nandamuri et al., 2017). *Andinoacara rivulatus* potentially represents one such species lacking the capacity for opsin expression plasticity in response to seasonal environmental changes.

It is also possible that the small number of individuals collected, precluded us to capture the full extent of variability present in the species. Additionally, the collected individuals may have specifically adapted to the particular light conditions of their local environment. As a result, the opsin expression patterns observed in this study might not reflect the broader variability that likely exists across the entire species. Some cichlids exhibit plasticity in opsin expression during specific developmental stages or in response to more

long-term environmental changes, rather than rapid seasonal fluctuations (Carleton et al., 2020). For this reason, future studies should take into account collecting individuals and environmental conditions every other day for one week within the same location (Costigan et al., 2015).

Furthermore, there might have been some experiment design limitations. The environmental conditions comparison and sample taking might have been insufficient. Individuals were collected form one site (Zancudo), environmental conditions from this area were measured only one time each season; this data possibly did not capture the environmental variation periods when opsin expression changes occur (Costigan et al., 2014). Finally, another possibility could be that the differences in light conditions between the two seasons may not have been substantial enough to trigger significant changes in opsin expression (Escobar-Camacho et al., 2019).

CONCLUSION

Andinoacara rivulatus did not exhibit opsin expression plasticity in light changing environment through the wet and dry season This species expressed a set of 5 different opsins. This support neotropical cichlid opsin research showing a reduced opsin expression profile sensitive to red, green, and blue light. *Andinoacara rivulatus* has a long wavelength shifted opsins in the visual system. Future research could explore other aspects of the visual system in *A. rivulatus*, such as the expression of other genes involved in phototransduction or neuronal processing, like the presence of vitamin A2 and its effect on cichlids vision; or the presence/absence of other opsin nonvisual types which might contribute to visual adaptation. Additionally, future research on opsin expression plasticity could focus on examining

different developmental stages or timescales, that could provide further insights into phenotypic plasticity in this species.

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APPENDIX A. MORPHOLOGICAL MEASUREMENTS FOR *ANDINOACARA RIVULATUS*

Individual	FL (mm)	TL (mm)	W(g)
A14	5.78	6.74	4.69
A15	8.75	10.25	16.97
A16	4.61	5.86	2.66
A17	4.07	5.05	1.85
A86	9.6	11.66	30.7
A87	8.22	9.88	15
A88	7.28	9.05	12.5
A90	6.84	8.5	7.1
A92	9.05	11.23	22.3