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Environmental enrichment increases the number of telencephalic but not tectal cells of angelfish (*Pterophyllum scalare*): an exploratory investigation using optical fractionator

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Abstract Based on previous evidence that environmental enrichment is associated with telencephalic cellular proliferation and that stable visuotopic tectal circuits are essential for discrimination of placement and identity of stationary or moving objects in the visual field, differential plasticity is expected in these areas. Here we tested this hypothesis in the Angelfish (*Pterophyllum scalare*), a species of ornamental fish with great value in the aquarist trade. We hypothesized that total telencephalic cell number would increase under the influence of an enriched environment whereas the tectal cell number would not change. To test this hypothesis, 12 aquaria of 80 l each were used, with six fish in each. The aquaria had either an enriched environment (EE) including stones, plants, sand and the presence of another fish from the Loricariidae family for interspecific social interaction, or an impoverished environment (IE), in which stimuli were limited to intraspecific interactions in a barren aquarium. After 62 days, six fish from each treatment were euthanized, and their brains were fixed and sectioned for Nissl staining. Then, stereological estimates of the total number of cells were performed. The fish showed no differences in weight gain, feed conversion ratio, condition factor, specific growth

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rate or survival. Animals kept in the enriched environment had a higher number of total telencephalic cells than animals kept in the impoverished environment (1,038,555 ± 65,357 vs. 758,331 ± 51,587, bilateral t-test, $p = 0.008$), but a similar number of tectal optical cells (EE 424,097 ± 29,914 vs. IE 471,409 ± 50,850, bilateral t-test, $p = 0.445752$). We concluded that cell proliferation in response to stimulation by the enriched environment is differentially expressed in the telencephalon and tectal areas of *Pterophyllum scalare*.

Keywords *Pterophyllum scalare* · Optic tectum · Telencephalon · Neuroplasticity · Stereology · Optical fractionator

Introduction

Neural cell proliferation is associated with the maintenance of complex neural mechanisms such as learning and memory (Abrous and Wojtowicz 2008; Ge et al. 2008; Perera et al. 2008), making the search for basic mechanisms associated with these functions essential for their understanding (Abrous and Wojtowicz 2008; Ge et al. 2008; Perera et al. 2008).

Captive animals in general exhibit lower performance in cognitive tests such as spatial memory and learning if adequate conditions for their maintenance are not met. In fact, intensive productive systems often pay no attention to the environmental factors necessary to meet the animals' requirements for adequate brain and physical development (Martins et al. 2012).

It is well known that sensory stimuli from the environment, such as those resulting from social interaction, physical exercise, and stressors, modulate neural cell proliferation in mammals (Gould et al. 1997; Kempermann et al. 1997; Van Praag et al. 1999; Eadie et al. 2005; Mirescu and Gould 2006), birds and fish.

In contrast to an impoverished environment (IE) that limits stimulation to social interaction, environments that provide diverse stimuli, including visuo-spatial stimulation, physical exercise and social interaction of individuals of the same and different species, are designated enriched environments (EE) (Rosenzweig et al. 1978).

In the present report we mimicked environmental enrichment by adding stones, plants, sand and an individual of the Loricariidae family (*Pterygoplichthys etentaculatus*) for interspecific social interaction to enriched aquaria, whereas the stimuli in impoverished aquaria were limited to intraspecific social interaction.

Although previous studies are not directly comparable, many of them investigated the influences of these two types of environment on neural mechanisms, especially in the cerebral cortex and the limbic system of mice (Kempermann et al. 1997; Branchi et al. 2006; Rossi et al. 2006; Schloesser et al. 2010), rats (Pham et al. 2002), zebrafish (von Krogh et al. 2010), *Brachyhypopomus gauderio* (Dunlap et al. 2011), *Oncorhynchus kisutch, Salmo salar* (Lema et al. 2005; Salvanes et al. 2013) and *C. auratus* (Abreu et al. 2019), demonstrating that the telencephalic cell cycle is influenced by environmental enrichment. From these analyses it has emerged that the sensory, motor and social stimuli of the enriched environment act on the brain by inducing plastic responses in the local and projection circuitry related to higher levels of neurotrophins and glial and neural plasticity, thereby increasing encephalic cellular proliferation in a variety of neurogenic niches (Pham et al. 2002; Branchi et al. 2006; Rossi et al. 2006; Zhu et al. 2006; Mora et al. 2007; Angelucci et al. 2009).

In teleost, neurogenesis occurs during adulthood and the rate of neuronal proliferation increases with age, body mass and length (Birse et al. 1980; Zupanc and Horschke 1995; Zupanc 2006; Zupanc 2008). However, little is known about how important the external influences on the neurogenesis of teleost are for local or projection circuits (von Krogh et al. 2010).

Pterophyllum scalare is one of the most important ornamental Teleostei species in the aquarist trade. Although *P. scalare* molecular genetics (Schneider et al. 2015; Li et al. 2016), reproduction and growth (Cacho et al. 2007; Ortega-Salas et al. 2009; Kacperczyk et al. 2011; Kasiri et al. 2012) have been investigated previously, and a number of studies have been dedicated to its behavior (Gómez-Laplaza and Morgan 2003, 2005; Barreto et al. 2006; Gómez-Laplaza 2009; Agrillo et al. 2012; Gómez-Laplaza and Gerlai 2015), only one study has attempted to quantify cells in the nervous system of this species (Sakamoto et al. 1999). Thus, potential correlations between environmental changes and telencephalic cell proliferation in this Amazon species in captivity are so far unknown. To search for differential cellular proliferative responses, we estimated in the same individuals the total number of cells in the superficial layers of the tectum opticum, a region that receives topographic retinal projections and sends its

axons topographically organized to other nuclei and areas of the central nervous system. Because no previous study has investigated the potential influences of environmental enrichment on the total number of telencephalon and tectal areas of Teleostei simultaneously with stereological methods, the objective of this work was to search for differential plasticity in these regions using an unbiased sampling approach (West 2002). The present study addressed this question while maintaining all other sources of potential variables, including water temperature, pH, O_2 concentration, day-light cycle, noise level, and number of individuals per volume of water in the aquaria, to minimize confounding factors.

Material and methods

All procedures in this study were previously approved by the ethics committee of UFPA/CEUA, n°1875240419. Fish were obtained from a natural spawning of a pair of marble Angelfish. The fingerlings were raised in an impoverished environment until the juvenile phase (0.5 g), and fed with artemia and commercial diet (32% crude protein) at 5% of live weight distributed among three feedings a day.

Seventy-two fish of the same age (mean ± SD; weight 0.5 ± 0.15 g and length 2.4 ± 0.13 cm) from the same spawning were distributed randomly and equally in 12 aquaria each with a capacity of 80 l and a recirculated water system. Two contrasting environments were used: an enriched environment (EE) and an impoverished environment (IE). In the EE aquaria there were stones, plants, sand and an individual of the Loricariidae family (*Pterygoplichthys etentaculatus*) for interspecific social interaction. In the IE aquaria there were no objects for visuospatial or interspecific stimulation, Fig. 1. The fish were accommodated in each aquarium in their juvenile form, and observed for 62 days. After 62 days of feeding fish performance was assessed to control other variables that may have influenced the results.

The results were then submitted to a t-test to detect significant differences at $p < 0.05$. To control for all other sources of potential variables that could affect the results, we measured weight gain (WG) (WG = final weight – initial weight); apparent feed conversion (AFC) (AFC = feed offered/WG); biomass (B) (B= $\sum$*fish weight*); weight uniformity (U) (U=N ± 20%/nt), where N is the number of fish weighing within 20% of the mean weight (Furuya et al. 1998); condition factor ($K=W/SL^b$), where W is the weight, SL is the standard length, and b is the angular coefficient obtained by weight-length regression (Le Cren 1951); and survival. Food was weighed and a standard amount was fed to each aquarium. Aquaria were not shielded from each other and the water was artificially aerated and filtrated. The water quality of each aquarium was monitored once a day, a natural photoperiod was adopted and the fish were fed a commercial diet (Poytara) twice a day at 3% of live weight. The water quality parameters were as follows: temperature 29.8 °C ± 0.12, OD 7.3 ± 0.16 mg/L and pH 8.3 ± 0.09 with no restriction on species growth.

After the experiment six fish from each treatment were euthanized using an overdose of Eugenol (clove oil, 0.40 mL/L) followed by craniectomy. The brains were dissected and immersed in 10% formalin before sectioning. The brains were cut in the axial plane using a vibratome (Leica VT1000S) into 70-μm-thick sections. Thereafter, the neurons and glial cells were stained with 0.5% cresyl violet, dehydrated and cleared with a series of alcohols and xylenes. Then, sections were embedded in Entellan® and coverslipped.

Stereological analysis

An optical fractionator (West et al. 1991) was used to estimate the total number of cells in the optic tectum and telencephalon of *Pterophyllum scalare*, Fig. 2. This method is independent of size, shape and cell orientation and is not affected by tissue retraction caused by histological procedures (West 2002). To start the optical fractionator procedures, the contours in the areas of interest in each section were defined using the StereoInvestigator (MBF Bioscience, Williston, VT, USA) software and the 4X objective lens of a NIKON Eclipse CI (Nikon, Japan) microscope equipped with a motorized stage (MAC6000, Ludl Eletronic Products, Hawthorne, NY, USA) for the X and Y axis and a linear transducer capturing information for the Z axis. The edges of the regions of interest were defined according to conspicuous changes identified in the patterns of the stained cells (for the optical tectum, the region of interest comprised the *stratum album central* (SAC), *stratum fibrosum et griseum superficiale* (SFGS) and *stratum griseum centrale*

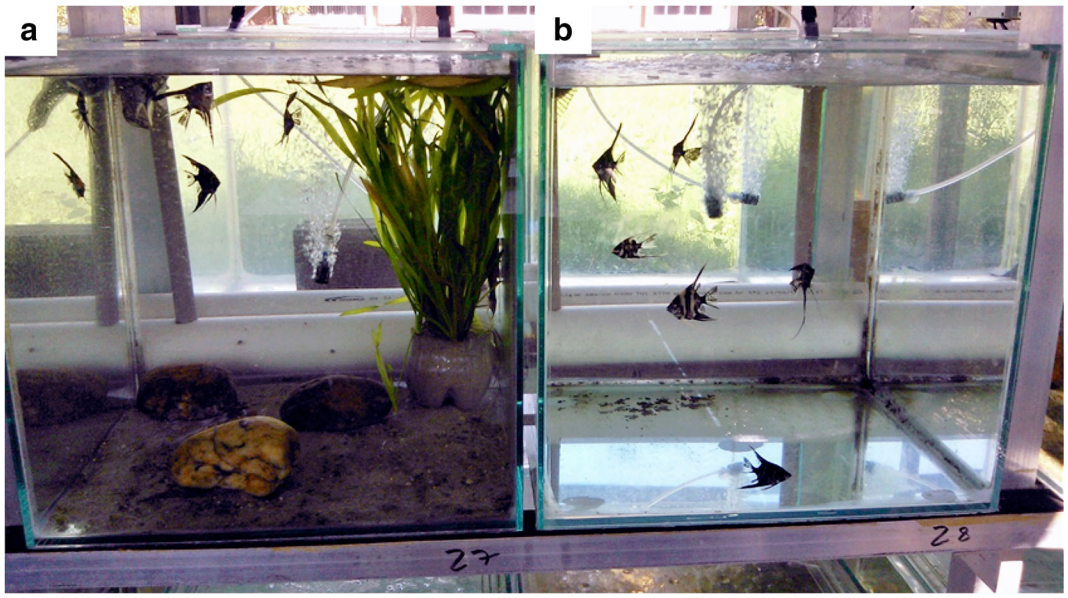

Fig. 1 Aquarium contrasting environments: (**a**) Enriched environment (EE); (**b**) Impoverished environment (IE)

(SGC), because they correspond to the layers that connect to the telencephalon (Meek 1983). For counting cells in the optical fractionator, an oil immersion, 100 x objective was used (Nikon, NA 1.45, WD = 0.13 μm). At each counting site, the thickness of the section was carefully assessed using the high-power objective and the fine focus of the microscope to define the adjacent defocused planes above (top of section) and below (bottom). The focused planes were digitized through analog-digital converters and stored using Stereoinvestigator software (MicroBright Systems

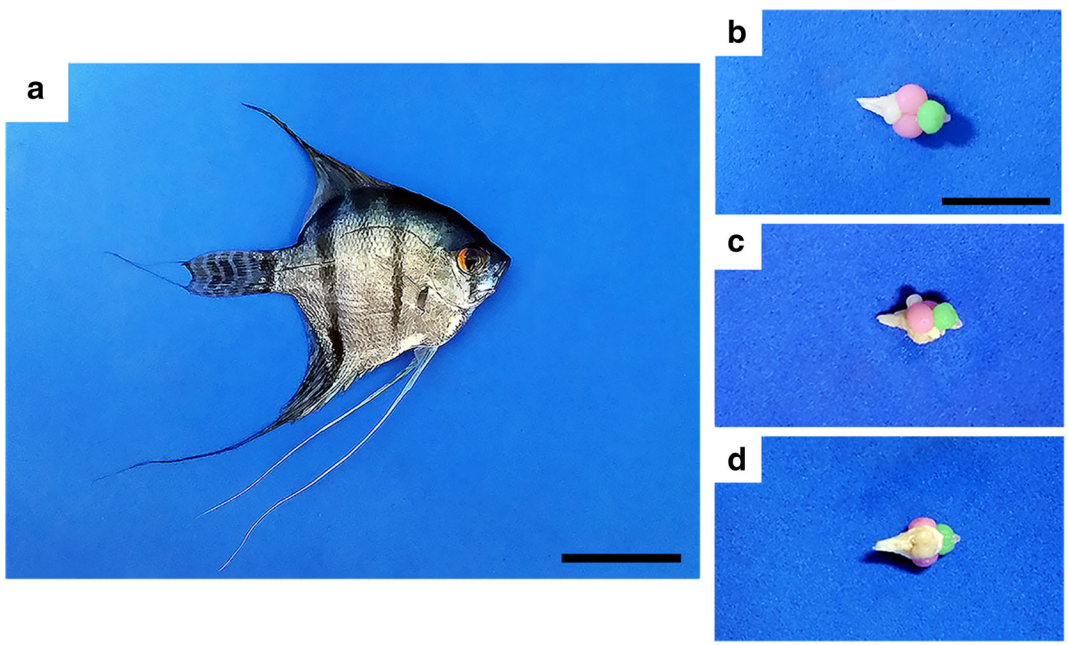

Fig. 2 Angelfish (*Pterophyllum scalare)* (**a**) Telencephalon (green) and optic tectum (pink) indicated by different colors in dorsal (**b**), lateral (**c**) and ventral view (**d**). Scale bars **a**: 3 cm; **b**, **c** and **d**: 1 cm

Inc.). Since the thickness of the post-dehydration section and the number of cells was heterogeneous throughout the counting sites, the estimated population used the number weighted section thickness. This estimate was used when thickness was measured at every sampling site and when the section thickness varied dramatically across the sections that included the region of interest. For a detailed explanation of the procedure, see https://www.mbfbioscience.com/help/si11/Content/SI_SPECIFIC/workflows/OF%20workflow/OF_ViewResults.htm.

In this procedure, all sampled cells should be in focus within the optical dissector or by intercepting the inclusion line of the counting box without intercepting the exclusion line (Gundersen and Jensen 1987). Estimates from the optical fractionator are obtained by multiplying the number of cells counted by the inverse of the sample fractions. The ssf (section sampling fraction) is the number of sections investigated compared to the total number of sections comprising the region of interest, asf (area sampling fraction) corresponds to the area of the sampled sections in relation to the total area that is organized in a grid (step x, y), and tsf (thickness sampling fraction) is defined by the height of the optical dissector in relation to the mean of the section thickness after the histological procedures. Therefore, the total number of cells is estimated for each marker using the following equation: $N = \Sigma Q \times 1/ssf \times 1/asf \times 1/tsf$, where N is the total number of cells and ΣQ is the number of cells counted (West et al. 1991).

The counting boxes are generated randomly and systematically according to a grid that determines the distances between the boxes Fig. 3. The size of the boxes and the grid are adjusted to increase the reliability of the sampling, and a coefficient of error is estimated for each set of selected parameters (Glazer and Wilson 1998).

The acceptable level of error for the reliability of the stereological estimates is defined by the ratio between the intrinsic error introduced by the methodology and the coefficient of variation. The variation introduced by the methodological procedures should be less than 50% of the total variance ($CE^2/CV^2 < 0.5$) (Slomianka and West 2005). Estimates of cell numbers were compared between treatments using a t-test (Table 1, Fig. 4).

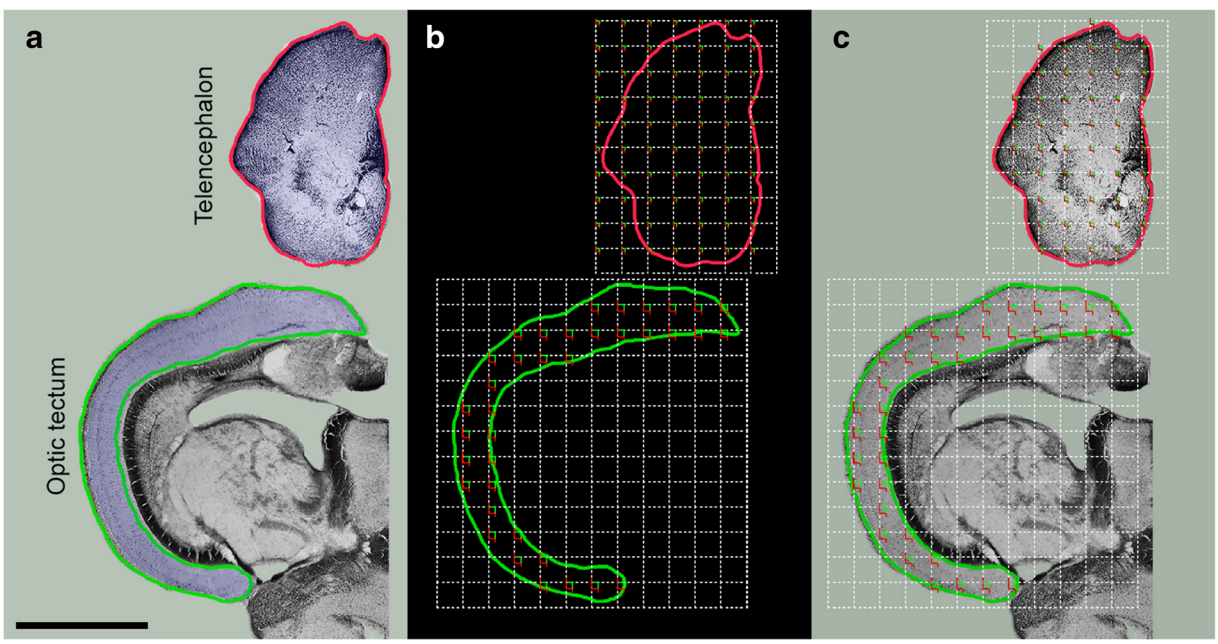

Fig. 3 Graphic representation of the systematic and random approach used to count telencephalic and tectal cells (**a**) axial section of sampled brain showing the regions of interest contoured by red (telencephalon) and green line (optic tectum) (**b**) the software StereoInvestigator use the contours to generate counting boxes then (**c**) each sampling site on the regions of interest defined by counting boxes is investigated. Scale bar: 1 mm

Table 1 Stereological parameters to count cells in the telencephalon and optic tectum of *Pterophyllum scalare*

Analyzed area	Animal	Frame (μm)	Grid (μm)	No. of frames	No. sections	SSF	ASF	TSF	ΣQ-
Telencephalon	IE26	25 × 25	200 × 200	115	4	0.167	0.016	0.704	1143
	IE28	25 × 25	200 × 200	136	5	0.167	0.016	0.735	1242
	IE30	25 × 25	200 × 200	133	4	0.167	0.016	0.765	1275
	IE31	25 × 25	200 × 200	109	4	0.167	0.016	0.735	1191
	IE33	25 × 25	200 × 200	171	5	0.167	0.016	0.777	2003
	IE35	25 × 25	200 × 200	131	4	0.167	0.016	0.769	3025
	EE25	25 × 25	200 × 200	107	4	0.167	0.016	0.620	1676
	EE27	25 × 25	200 × 200	109	4	0.167	0.016	0.636	1202
	EE34	25 × 25	200 × 200	125	5	0.167	0.016	0.584	1655
	EE36	25 × 25	200 × 200	95	4	0.167	0.016	0.547	1500
	EE29	25 × 25	200 × 200	122	5	0.167	0.016	0.579	1762
	EE32	25 × 25	200 × 200	118	5	0.167	0.016	0.540	1622
Optic tectum	IE30	50 × 50	200 × 200	156	5	0.167	0.062	0.562	2882
	IE33	50 × 50	200 × 200	163	5	0.167	0.062	0.554	3279
	IE28	50 × 50	200 × 200	157	5	0.167	0.062	0.568	2974
	IE26	50 × 50	200 × 200	158	6	0.167	0.062	0.449	2678
	IE31	50 × 50	200 × 200	143	5	0.167	0.062	0.562	2668
	IE35	50 × 50	200 × 200	62	4	0.167	0.062	0.556	1360
	EE34	50 × 50	200 × 200	133	5	0.167	0.062	0.505	2466
	EE36	50 × 50	200 × 200	120	4	0.167	0.062	0.379	1524
	EE29	50 × 50	200 × 200	117	4	0.167	0.062	0.449	1737
	EE32	50 × 50	200 × 200	118	4	0.167	0.062	0.497	1753
	EE25	50 × 50	200 × 200	137	5	0.167	0.062	0.493	2769
	EE27	50 × 50	200 × 200	120	4	0.167	0.062	0.612	2806

ΣQ- Equals the total number of objects of interest counted using the optical dissector, *SSF* Section Sampling Fraction, *ASF* Area Sampling Fraction, *TSF* Thickness Sampling Fraction, *Frame* Counting frame size, *Grid* Grid size

Results

Table 2 summarizes all fish parameters used as controls of other potential variables that may affect telencephalic cell counts. Compared with fish in the IE, fish in the EE had a higher number of telencephalic cells (1,038,555 ± 65,357 vs. 758,331 ± 51,587, bilateral t-test, $p = 0.008316$), but there was no significant difference in the number of optic tectum cells (EE 424,097 ± 29,914 vs. IE 471,409 ± 50,850, bilateral t-test, $p = 0.445752$) (see Tables 2 and 3).

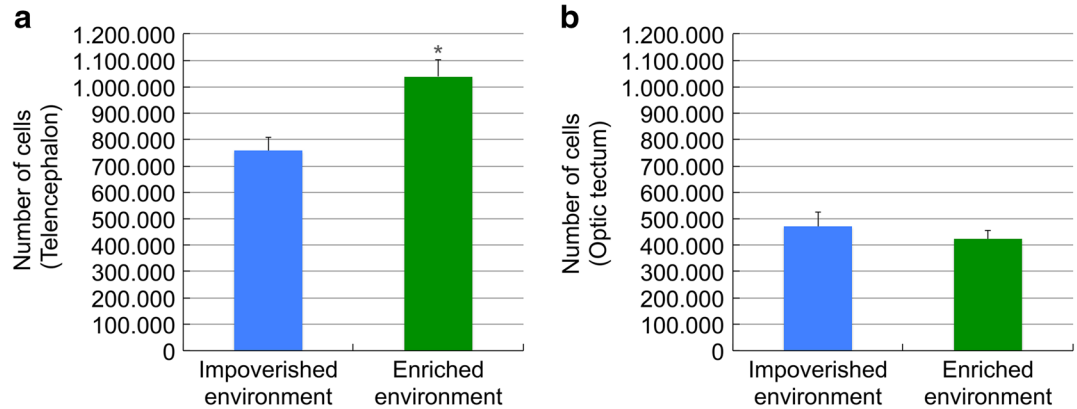

Fig. 4 Stereological results of total cell counts in *Pterophyllum scalare*: Telencephalon (**a**); Optic tectum (**b**)

Table 2 Telencephalic stereological results

Telencephalon	Number cells	Thickness	CE Scheaffer	Volume (mm^3)	Cells/mm^3
IE26	673,003.63	21.3	0.067	1.90	354,212.4368
IE28	755,349.50	20.4	0.057	2.4	314,728.9583
IE30	704,404.38	19.6	0.045	2.1	335,430.6571
IE31	690,060.06	20.4	0.048	1.7	405,917.6824
IE33	1,009,905.88	19.3	0.041	2.8	360,680.6714
IE35	717,262.19	19.5	0.050	2.0	358,631.095
Mean	758,331.00	20.0833333	0.051	2.15	354,933.5835
Standard deviation	126,360.768	0.75740786	0.00935236	0.39370039	30,474.16946
CV	0.16663011				
CV^2	0.027765594				
CE^2	0.002635111				
CE^2/CV^2	0.094905629				
CE^2 - CV^2	0.025130482				
CBV^2(%)	90.50943714				
EE25	1,036,789	24.2	0.052	1.8	575,994.1
EE27	730,104	23.6	0.056	1.8	405,613.5056
EE34	1,089,341	25.7	0.05	2.0	544,670.375
EE36	1,052,720	27.4	0.071	1.5	701,813.5
EE29	1,169,723	25.9	0.05	1.9	615,643.4895
EE32	1,152,653	27.8	0.051	1.9	606,659.4105
Mean	1,038,555	25.76666667	0.055	1.816666667	575,065.7301
Standard deviation	160,092.5143	1.671725655	0.00814862	0.172240142	98,334.64116
CV	0.154149284				
CV^2	0.023762002				
CE^2	0.003025				
CE^2/CV^2	0.12730409				
$CE^2 – CV^2$	0.020737002				
CBV^2(%)	87.26959097				

CV Coefficient of variation, *CE* Coefficient of error, *CVB* Coefficient of Biologic Variation

Discussion

We demonstrated the influence of environmental enrichment on the number of cells in the telencephalon of *Pterophyllum scalare*. The comparative analysis of the total cell numbers of the telencephalon between the groups, using cresyl violet, corroborates previous studies done with immunological markers of cell proliferation, with increased cell numbers in the enriched environment (von Krogh et al. 2010).

Neural cell proliferation increases with age, length and body mass in teleosts (Birse et al. 1980; Zupanc and Horschke 1995; Zupanc 2006; Zupanc 2008). There was no difference in fish growth between the EE and IE group, but the EE group showed more fish weight uniformity. We found a significantly higher number of telencephalic cells in the EE than in the IE group. A previous study in *Danio rerio* showed that compared with an impoverished environment, individuals from an enriched environment exhibited a reduction in locomotor activity and a smaller increase in cortisol, resulting in greater proliferative activity of the telencephalic cells (Von Krogh et al. 2010).

Our findings are consistent with previous descriptions in both *Danio rerio* and *C. auratus* (von Krogh et al. 2010; Abreu et al. 2019) and showed the differential effects of environmental enrichment on the

Table 3 Optic tectum stereological results

Optic tectum	Number cells	Thickness	CE Scheaffer	Volume (mm^3)	Cells/mm^3
IE30	493,366	26.7	0.046	2.1	230,545
IE33	569,018	27.1	0.040	2.3	247,399
IE28	503,136	26.4	0.040	2.2	228,698
IE26	572,697	33.4	0.050	2.0	286,348
IE31	455,566	26.7	0.046	2.0	227,783
IE35	234,672	27.0	0.060	0.9	260,746
Mean	471,409	27.9	0.047	1.9	246,920
Standard deviation	124,556	2.71	0.007	0.51	23,291
CV	0.264220538				
CV^2	0.069812492				
CE^2	0.002209				
CE^2/CV^2	0.031641901				
$CE^2 - CV^2$	0.067603492				
CBV^2(%)	96.83580986				
EE34	469,162	29.7	0.044	2.1	223,410
EE36	386,670	39.6	0.051	1.6	241,669
EE29	371,812	33.4	0.057	2.0	185,906
EE32	338,823	30.2	0.049	1.6	211,764
EE25	538,903	30.4	0.040	1.9	283,633
EE27	439,214	24.5	0.049	1.6	274,509
Mean	424,097	31.3	0.048	1.8	232,539
Standard deviation	73,275	4.98	0.006	0.23	37,511
CV	0.172778481				
CV^2	0.029852403				
CE^2	0.002336111				
CE^2/CV^2	0.078255378				
CE^2 - CV^2	0.027516292				
CBV^2(%)	92.17446223				

CV Coefficient of variation, *CE* Coefficient of error, *CVB* Coefficient of Biologic Variation

number of cells of the optical tectum and of the telencephalon, suggesting differential neuroplasticity of these two regions in response to environmental changes.

In contrast to endotherms, ectothermic animals exhibit cell proliferation due to nonspecific influences of the environment, such as changes in body temperature (Rieder and Cole 2002; Radmilovich et al. 2003), sex (Perry and Grober 2003), age (Zikopoulos et al. 2000; Traniello et al. 2014), or somatic and neural injuries followed by regeneration (Ilieş et al. 2014). Either in isolation or in combination these effects alter cell counts (Dunlap 2016). In the present report we minimized these potential influences by controlling the number of individuals per volume of water, water temperature, pH, O_2 concentration, day-light cycle, noise level, sex, and age in both environmentally enriched and impoverished aquaria. Thus, we expect that the significant differences found between total telencephalic cell estimates in individuals maintained in environmentally enriched and impoverished aquaria are rather specific and associated with the contrasting environments. Although we did not investigate the subjacent mechanisms in the present study, we extended to *P. scalare* previous observations on telencephalic cell proliferation in the forebrain of zebrafish (von Krogh

et al. 2010), *Brachyhypopomus gauderio* (Dunlap et al. 2011), *Oncorhynchus kisutch* and in the *Salmo salar* species (Lema et al. 2005; Salvanes et al. 2013) and *C. auratus* (Abreu et al. 2019), demonstrating that the cell cycle in the telencephalon is also altered by environmental enrichment.

It is important to point out that, we used different objects to enrich the aquaria and that different objects may interact with and add or subtract beneficial effects on cellular proliferation. However, because the interactions between individuals were potentially similar in both types of aquaria, it is reasonable to suggest that visuospatial stimulation and interspecific interaction in the enriched environment may be the experimental variables that were associated with a higher number of cells in the telencephalon. In addition, no significant differences were found between body size or body weight between individuals from enriched and impoverished environments, suggesting that significant differences in cell counts were not associated with potential differences in the growth rate or body weight.

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Availability of data and material The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Author's contributions All authors listed executed substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; and final approval of the version to be published; agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Conflict of interest The authors have no any conflict of interest to declare.

Ethics approval Approved by the ethics committee of UFPA/CEUA, n°1,875,240,419.

Consent to participate Not applicable.

Consent for publication The authors agree with this publication.

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