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COMPARATIVE NEUROANATOMY AND ECO-BEHAVIOR INFERENCES IN SPECIES OF ANABLEPS SCOPOLI, 1997 (TELEOSTEI: CYPRINODONTIFORMES)

NEUROANATOMIA COMPARADA E INFERÊNCIAS ECOLÓGICO-COMPORTAMENTAIS EM ESPÉCIES DE ANABLEPS SCOPOLI, 1997 (TELEOSTEI: CYPRINODONTIFORMES)

NEUROANATOMÍA COMPARADA E INFERENCIAS ECOLÓGICO-COMPORTAMENTALES EN ESPECIES DE ANABLEPS SCOPOLI, 1997 (TELEOSTEI: CYPRINODONTIFORMES)

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ABSTRACT:

The study comparatively analyzes the neuroanatomy of two *Anableps* species with the aim of evaluating the limits of brain plasticity and investigating whether ecological and behavioral differences may be reflected in the macroanatomy of the nervous system. This is a descriptive and comparative study that examined adult specimens of *Anableps anableps* and *Anableps microlepis*, collected along the coast of Pará, through neurocranial dissections, morphometric measurements, and volumetric analysis of the main encephalic structures. The results reveal that the brain of *Anableps* exhibits similarity to the generalized pattern described for Teleostei, but with particular features related to its sensory specialization. The main differences between the species are located in the *telencephalon* and *rhombencephalon*. The identified variations suggest that differences in migratory behavior and preferences for distinct habitats may have shaped the encephalic morphology of the taxa. This study demonstrates that phylogenetically related species may exhibit significant macroanatomical modifications associated with their mode of life, highlighting the role of phylogenetic plasticity and adaptive pressures in the evolution of the fish brain. The findings expand knowledge of neuroanatomical diversity within the genus *Anableps* and the order Cyprinodontiformes.

KEYWORDS: Tralhoto; Four-eyed fish; brain; encephalon.

RESUMO:

O trabalho analisa comparativamente a neuroanatomia de duas espécies de *Anableps* com o objetivo de avaliar os limites da plasticidade do encéfalo e investigar se diferenças ecológicas e comportamentais podem refletir-se na macroanatomia do sistema nervoso. Trata-se de um estudo descritivo e comparativo que examinou exemplares adultos de *Anableps anableps* e *Anableps microlepis*, coletados na costa do Pará, por meio de dissecções do neurocrânio, mensurações morfométricas e análise volumétrica das principais estruturas encefálicas. Os resultados revelam que o encéfalo de *Anableps* apresenta semelhança com o padrão generalizado descrito para Teleostei, mas com particularidades relacionadas à sua especialização sensorial. As principais diferenças entre as espécies localizam-se no *Telencephalon* e *Rhombencephalon*. As variações identificadas sugerem que diferenças no comportamento migratório e na preferência por habitats distintos podem ter moldado a morfologia encefálica dos táxons. Este estudo demonstra que espécies filogeneticamente relacionadas podem apresentar modificações macroanatômicas expressivas associadas ao modo de vida, destacando o papel da plasticidade filogenética e das pressões adaptativas na evolução do encéfalo dos peixes. Os achados ampliam o conhecimento sobre a diversidade neuroanatômica no gênero *Anableps* e da ordem Cyprinodontiformes.

PALAVRAS-CHAVE: Tralhoto; Peixe-de-quatro-olhos; cérebro; encéfalo.

RESUMEN:

El trabajo analiza comparativamente la neuroanatomía de dos especies de *Anableps* con el objetivo de evaluar los límites de la plasticidad del encéfalo e investigar si las diferencias ecológicas y comportamentales pueden reflejarse en la macroanatomía del sistema nervioso. Se trata de un estudio descriptivo y comparativo que examinó ejemplares adultos de *Anableps anableps* y *Anableps microlepis*, recolectados en la costa de Pará, mediante disecciones del neurocráneo, mediciones morfométricas y análisis volumétrico de las principales estructuras encefálicas. Los resultados revelan que el encéfalo de *Anableps* presenta similitudes con el patrón generalizado descrito para Teleostei, aunque con particularidades relacionadas con su especialización sensorial. Las principales diferencias entre las especies se localizan en el *Telencephalon* y el *Rhombencephalon*. Las variaciones identificadas sugieren que las diferencias en el comportamiento migratorio y en la preferencia por distintos hábitats pueden haber moldeado la morfología encefálica de los taxones. Este estudio demuestra que especies filogenéticamente relacionadas pueden presentar modificaciones macroanatómicas significativas asociadas al modo de vida, destacando el papel de la plasticidad filogenética y de las presiones adaptativas en la evolución del encéfalo de los peces. Los hallazgos amplían el conocimiento sobre la diversidad neuroanatómica en el género *Anableps* y en el orden Cyprinodontiformes.

PALABRAS CLAVE: Tralhoto; pez de cuatro ojos; cerebro; encéfalo.

INTRODUCTION

The order Cyprinodontiformes encompasses a diverse group of fishes popularly known as killifishes or livebearers. Distributed across 14 families, 152 genera, and 1,418 species, they are found primarily in tropical and subtropical regions (Baumgartner et al., 2012; Nelson, Grande, Wilson, 2016; Fricke, Eschmeyer, van der Laan, 2025), inhabiting environments ranging from freshwater rivers and ponds to brackish and coastal zones, including extreme hypersaline, hypoxic, or temporary habitats (Domínguez-Castañedo et al., 2013; Riesch, Tobler, Plath, 2015). The order is also notable for its diversity of reproductive strategies and frequent sexual dimorphism (Turner, 1938). Thus, considering its ecological range and taxonomic diversity, the group represents a vast field for evolutionary studies.

The largest Cyprinodontiformes are the so-called *tralhotos* or four-eyed fish, names given to species of *Anableps*. Individuals can reach a maximum total length (TL) of 320 mm, with females generally being larger than males (Nelson, Grande, Wilson, 2016). The term derives from the Greek *anablepein*; 'ana' (up) and 'blepein' (to look) (Merriam-Webster Dictionary, 2025), in reference to the morphology of their eyes. Three species comprise the genus: *Anableps anableps* Linnaeus, 1758, and *Anableps microlepis* Müller & Troschel, 1844, which are sister and sympatric species, and *Anableps dowi* Gill, 1861, considered a basal group within the genus (Amorim, Costa, 2018) . The first two are distributed from the Gulf of Paria in Venezuela to the Parnaíba River delta in Piauí, Brazil (Oliveria, 1974; Cervigón et al., 1992). *A. dowi* occurs on the Pacific coast, from southern Mexico to Nicaragua (Miller, 1979).

Four-eyed fish are euryhaline, benthopelagic, and typically live in schools (Turner, 1938; Miller, 1979). They can occasionally be observed emerging to capture terrestrial invertebrates near tree roots in mangroves, an amphibious habit common to several species of Cyprinodontiformes (Brenner, Kumme, 2007; Turko, Wright, 2015). The particular anatomy of their modified eyes is a subject of great interest to scientists. *Anableps* possesses an expanded and functionally duplicated cornea, pupil, and retina, allowing for simultaneous vision of both aquatic and aerial environments (Oliveira, Fontoura, Montag, 2011). Consequently, the animal feeds on aquatic organisms such as algae and small fish within the water, as well as on intertidal, terrestrial, and even low-flying aerial prey outside of it (Miller, 1979; Götz, Greven, Kervath, 2001; Figueiredo et al., 2019). Their foraging is highly influenced by and adapted to tidal cycles, leading them to adopt intertidal migratory behavior (Brenner, Kumme, 2007).

Ecological and behavioral adaptations can be studied in light of the evolution of the Central Nervous System (CNS). The CNS evolved to enable animals to survive in distinct environments, diversifying across lineages (Ito et al., 2007; Vernier, 2017). Authors such as Ito et al. (2007), based on comparative neuroanatomy, proposed that the diversity of forms found in the teleost brain results from the genetic potential generated by gene duplication, coupled with evolutionary adaptations to different ecological niches. According to the authors, the diversity found in the teleost brain is unparalleled in other vertebrate groups, particularly when considering structures responsible for the integration and processing of sensory information derived from the alar plate region. It is believed that a whole-genome duplication occurred at the base of the Actinopterygii lineage, an event that likely amplified the occurrence of mutations and the genetic diversity observed in teleosts (Amores et al., 1998). For Ito et al. (2007), germline mutations in the brain's morphogenetic program, responsible for structural modifications, somehow accompanied changes in the corresponding sensory organs, thereby delimiting different ecological niches.

Dunlap (2016) sought to explain how the brain is capable of remodeling itself throughout the lifespan while maintaining high phenotypic plasticity. According to the author, conserved rates of neurogenesis in adulthood, a hallmark of the CNS in teleost fishes, allow for rapid, nonspecific structural and functional adjustments in the brain in response to general physiological changes within the organism. Conversely, when alterations in cell proliferation occur in specific brain structures, there may be a functional correspondence with behavioral changes in the fish. Other studies, such as those by van Staaden et al. (1995), Huber et al. (1997), Evans (1997), Ito et al. (2007), Pollen et al. (2007), Kotrschal et al. (2012), Ching, Senoo, and Kawamura (2014), and Oliveira and Graça (2024), have sought to understand how the environment and behavior can shape the fish brain.

Although studies on the morphological and structural diversity of the nervous system in teleost fishes have advanced considerably in recent decades, representing significant progress in the understanding of brain evolution and plasticity, many aspects of nervous system diversity remain underexplored, and the limits and mechanisms of this organ's plasticity are not yet fully understood (Kotrschal et al., 2012; Fong et al., 2019). In this context, the present study aims to contribute to the understanding of brain plasticity by comparing the macroanatomy of two phylogenetically related species, recording variations between the taxa and investigating possible associations with ecological and behavioral aspects. Thus, we aim to discuss whether changes in the species' lifestyle may be associated with macroanatomical variations in the Central Nervous System.

METHODOLOGY

To carry out the study, the neurocrania of the species *A. anableps* and *A. microlepis* were dissected to expose the brain and analyze neural regions and structures. Descriptive and morphometric data were collected and used for interspecific comparison. Ecological and ethological inferences were based on previous studies addressing the relationship between ecology, behavior, and brain morphology in teleost fishes. Furthermore, the research does not include field behavioral or ecological analyses, relying instead on literature reviews to understand the behavior and ecology of the four-eyed fish.

Material examined

A total of 14 adult specimens of *A. microlepis* (7 males and 7 females; 115–130 mm SL) and 6 adult specimens of *A. anableps* (4 males and 2 females; 115–200 mm SL) were examined. The *A. microlepis* specimens were collected on Algodoal Island, in the state of Pará, while the *A. anableps* specimens were obtained from the coast of Salinópolis, Pará. The material was fixed and stored in 70% ethanol.

Preparation and dissection procedures

Dissection was preceded by a preparation stage, which included obtaining body measurements and staining the specimens. Measurements followed Weitzman (1979) and were taken using a digital caliper with 0.01 mm precision and a scale for weighing. Specimen staining was based on the muscle dissection protocol described by Datovo and Bockmann (2010), employing a double-staining technique to highlight bone sutures and cartilage.

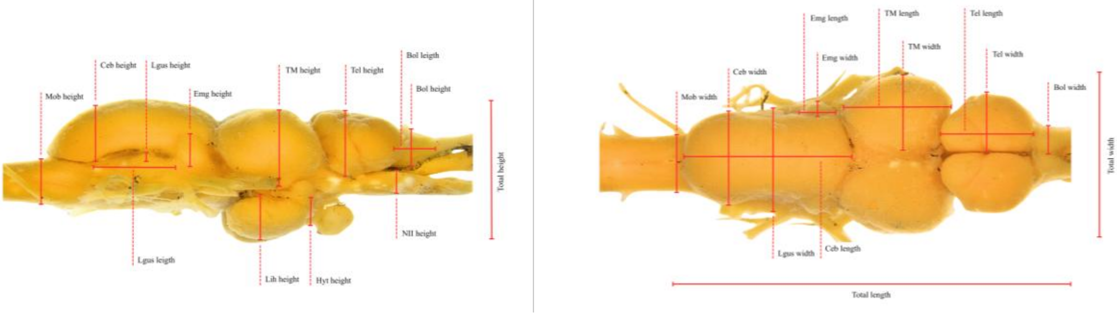
After this, the animals are submerged in 70% ethanol, and the specimens were dissected with the aid of a stereomicroscope. To standardize the neurocranium dissection process, the protocols proposed by Pereira and Castro (2016) for Characiformes and the neurocranium dissection protocol for Siluriformes by Abrahão and Pupo (2014) were used. Adaptations to the mentioned protocols were necessary as this involves a distinct order, given the absence of a specific dissection protocol for the Cyprinodontiformes neurocranium and considering the significant variation in the arrangement of the cranial roof bones.

The main procedures adopted for the dissection of the neurocranium of *Anableps* representatives are listed below. Initially, the epidermal layer is scraped to remove scales and the skin itself from the mesethmoid to the supraoccipital, to facilitate the visualization of bone sutures. Next, using a scalpel, incisions are made at the convergence of the posterior ends of the frontal bones and the anterior portion of the supraoccipital, sequentially tracing the sutures of the supraoccipital with the parietals, epiotics, and epiotic processes, to allow for the partial or complete removal of the supraoccipital and exposure of the posterior region of the *tectum mesencephali*. Subsequently, the remaining adjacent cranial roof bones are removed using a plier, followed by the removal of the lateral otoliths. Finally, transverse sections of the lateral cranial nerves and the olfactory and optic nerves are performed to carefully remove the brain from the cranial floor using forceps.

Brain Measurements and Statistical Analysis

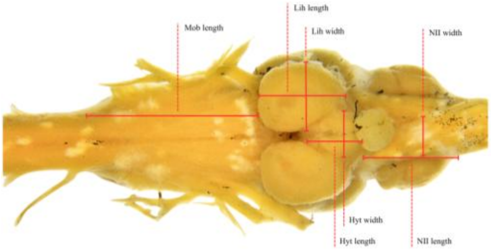
To quantitatively characterize the *Anableps* brain, a stereomicroscope with an attached Leica MC160 HD camera and an analytical balance for weighing were used. The brains were immersed in 70% alcohol gel to maintain their position and prevent displacement, and then fully submerged in liquid 70% alcohol to avoid image distortion caused by the gel. Following Pollen et al. (2007), 32 linear measurements of the brain were obtained (Figure 01).

Figure 01. Linear measurements of the brain. Bol: *bulbus olfactorius*; Ceb: *cerebellum*; Emg: *eminentia granularis*; Hyt: *hypothalamus*; Lgus: *lobus gustativus*; Lih: *lobus inferior hyphotalami*; Mob: *medulla oblongata*; NII: *nervus opticus*; Tel: *telencephalon* and TM: *tectum mesencephali*.



(A): *Lateral view*

(B): *Dorsal view*



(C): *Ventral view*

Source: Authors, 2026.

The total brain volume (Vt) was estimated using the formula Density = Mass/ Volume, assuming a brain tissue density of 1.036 mg/mm³ (Stephan, 1960). The volume (V) of each brain structure was estimated based on an ellipsoid model. The formula for half an ellipsoid was used for structures such as the *telencephalon* and *bulbus olfactorius*:

$$V = (\text{Length x Height x Width})\ \tfrac{1}{6}\ \pi$$

For structures such as the *medulla oblongata* and *cerebellum*, whose shapes do not resemble an ellipse, the formula for a quarter of an ellipsoid was used:

$$V = (\text{Length x Height x Width})\ \tfrac{1}{3}\ \pi$$

The relative volume (Vr) of the structures, calculated using the formula Vr = (V/Vt) x 100, was used for comparative analyses.

To identify differences in the relative size of brain structures between species, Student's t-test for independent samples was used when the assumptions of normality and homogeneity of variances were met, as determined by the Shapiro-Wilk and Levene tests, respectively. For groups exhibiting heterogeneous variances, Welch’s test was applied to correct for this violation and provide a more robust estimate of the difference between means. Statistical analyses were conducted using R software (R Core Team, 2025).

Anatomical Nomenclature

The anatomical directional terminology and the nomenclature of CNS structures and regions used in the present study are based on the conventions described by the *Nomina*

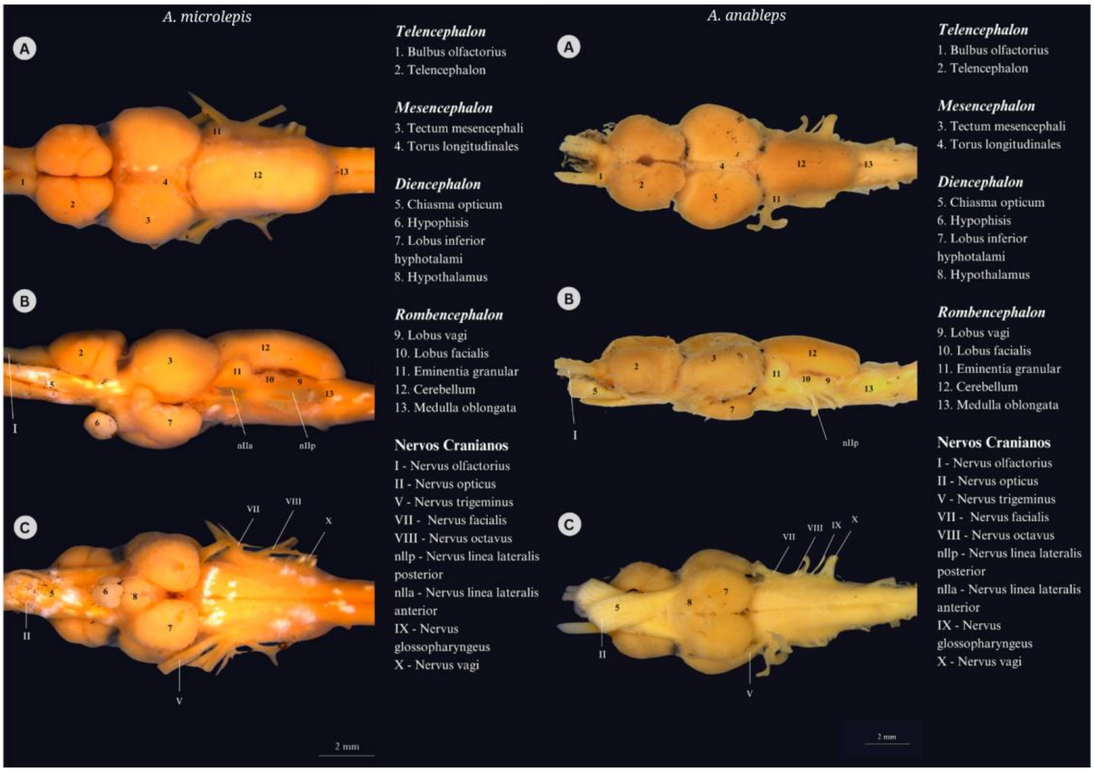
Anatomica Veterinaria (ICVGAN, 2017) and Nieuwenhuys et al. (1998), the latter being a classic reference in fish comparative neuroanatomy.

RESULTS AND DISCUSSION

General Description of the Gross Anatomy of the Brain in *Anableps*

Overall, the brain of *Anableps* is similar to the generalized teleost brain proposed by Meek and Nieuwenhuys (1998) based on the species *Oncorhynchus mykiss* Walbaum, 1792 (Salmoniformes). The rostral portion of the brain is located immediately beneath the frontal bones of *Anableps*. These bones, being expanded to accommodate the modified eyes (Parenti, 1981), cover a large part of the *Mesencephalon*. Its shape is long and relatively flattened, and it may present whitish spots, most frequently on the *medulla oblongata*, *truncus encephali*, and *nervus opticus* (Figure 02). A dense lipid layer is found between the meninges and the cranial roof.

Figure 02. Main brain regions and associated structures. *A. microlepis* specimen (180 mm SL) and *A. anableps* specimen (175 mm SL). A: dorsal view; B: lateral view; C: ventral view.



Source: Authors, 2026.

The *Rhombencephalon* exhibits a *medulla oblongata* that maintains a cylindrical shape throughout its length, expanding moderately in a caudorostral direction. A horizontal sulcus runs dorsally and ventrally along the structure, extending from the transition region between the *medulla oblongata* and the *medulla spinalis* to the *truncus encephali*. Positioned dorsally relative to the *medulla oblongata*, the *lobus vagi* and *lobus facialis* are covered by the elongated cerebellar corpus. The *lobus vagi* is a paired structure with a semicircular shape and medial ends that are not coapted. Adjacent to this lies the *lobus facialis*, also a paired structure. In this region, the horizontal sulcus of the *medulla oblongata* undergoes an abrupt expansion, separating the right and left pairs of gustatory lobes and expanding caudorostrally. Still within the *Rhombencephalon* region, the *cerebellum*, positioned dorsally, possesses a semi-uniform rectangular configuration and may present a central constriction of varying degree on the lateral walls. These, in turn, are adjacent to the *eminentia granularis*, a spherical, paired structure positioned anterolaterally to the cerebellar corpus.

The *Mesencephalon* displays a paired *tectum mesencephali* with an ovoid shape and a smooth surface. The *torus longitudinalis* is interposed medially between the right and left pairs of the *tectum mesencephali* and has an elongated, non-spherical form. The *nervus opticus* originates from the anteroventral portion of the *tectum mesencephali*, exhibits a distinct fringed appearance, and maintains a notably greater thickness relative to the other cranial nerves. Its width accounts for, on average, 20 to 25% of the total width of the brain.

The *Diencephalon* is located immediately beneath the *Mesencephalon*. In this region, the *lobus inferior hypothalami* constitutes a paired structure, which may present rectilinear or rounded faces depending on the species analyzed. Its medial ends, however, remain invariably rectilinear. Positioned anteromedially to the *lobus inferior hypothalami* is the *hypothalamus*, a bipartite structure that exhibits a central indentation. Its relationship with the *hypophysis* occurs in an anteroventral position. The *hypophysis* has a spherical shape, and its connection to the *hypothalamus* is relatively fragile and easily broken.

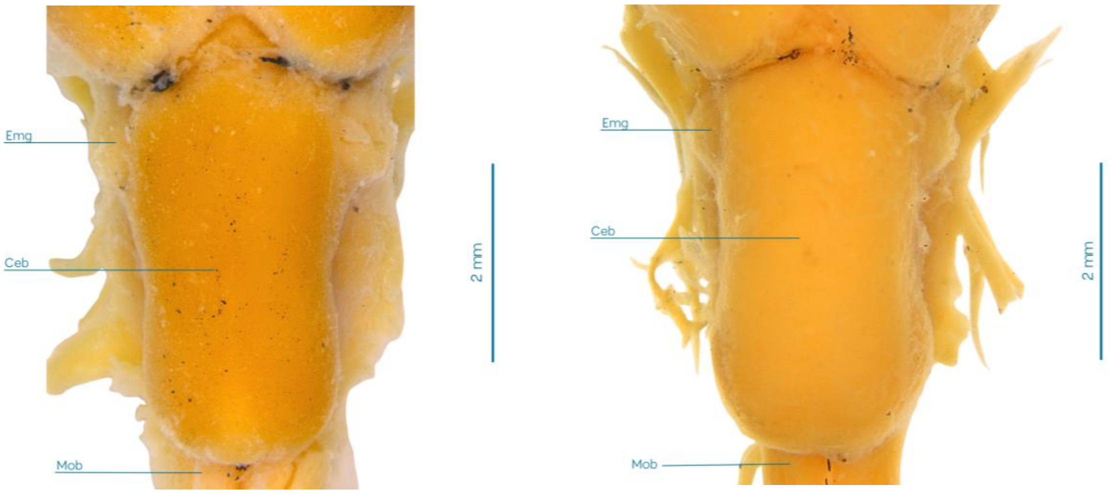
In the *Telencephalon*, we find the telencephalon proper, which possesses a reniform shape and an irregular surface. The dorsal region, corresponding to the *pallium*, exhibits a discreet posterior projection, while in the ventral region, termed the *subpallium*, this projection is more pronounced. The most rostrally located structure is the *bulbus olfactorius*, which is paired and semi-ovoid in shape. Its posterior portion connects anteroventrally to the *telencephalon*, which partially covers it when observed in dorsal view.

Comparison between *A. anableps* and *A. microlepis*

The most significant variations are concentrated in the *Prosencephalon*, composed of the *Telencephalon* and the *Diencephalon*, and in the *Rhombencephalon*, the most caudal region of the brain, frequently referred to as the “primitive brain”.

In the *Rhombencephalon*, the observed differences are concentrated in the *cerebellum* and the *lobus gustativus*. In *Anableps*, the *cerebellum* is an elongated structure with a semi-rectangular shape and a central constriction. For *A. anableps*, the central constriction appears to be more pronounced than in *A. microlepis*, such that the gustatory area, located immediately below the *cerebellum*, becomes more laterally evident (Figure 03). This observation may be related to the size of the *cerebellum* between the two species. The relative volume of the structure ranged from 10.5% to 32.8% (Graph 01), corresponding, respectively, to the lowest values in *A. anableps* and the highest in *A. microlepis*. Statistical analysis indicated a significant difference between the species; therefore, the *cerebellum* of *A. microlepis* presents a notably higher relative volume than that of *A. anableps* ($p < 0.001$). On the other hand, the *eminentia granularis*, a structure anterolaterally adjacent to the *cerebellum*, showed greater development in *A. anableps* ($p < 0.002$).

Figure 03. Comparison of the dorsal view of the *cerebellum* in the brain across species. Ceb: *cerebellum;* Emg: *eminentia granularis;* Lgus: *lobus gustativus* and Mob: *medulla oblongata*.

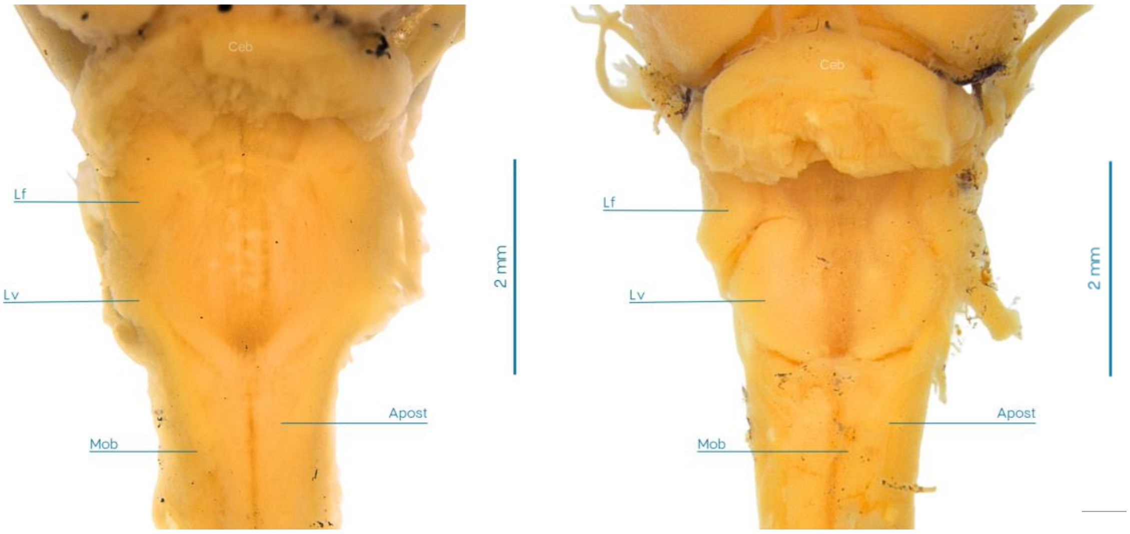


(A): *A. anableps* (B): *A. microlepis*

Source: Authors, 2026.

As for the *lobus gustativus*, it is possible to find inconspicuous projections and boundaries for the *lobus vagi*, as well as a more elliptical shape for the *lobus facialis* in *A. anableps*. In *A. microlepis*, the *lobus vagi* has a lunulate shape and clearer boundaries (Figure 04). Its anterolateral extremity converging at the midline, for example, is a striking borderline zone. The *lobus facialis* in *A. microlepis* has a triangular shape, with edges that are more rectilinear than rounded, as seen in *A. anableps*. There appears to be a slight inversion regarding the robustness of the gustatory lobes between the species. In *A. anableps*, the *lobus facialis* is more prominent, while the *lobus vagi* is less developed; in contrast, in *A. microlepis*, the *lobus facialis* is less prominent and the *lobus vagi* appears more developed.

Figure 04. Comparison of the dorsal view of the *lobus gustativus* in the brain across species. Apos: *area postrema* Ceb: *cerebellum*; Emg: *eminentia granularis*; Lf: lobus faciales; Lv: *lobus vagi* and Mob: *medulla oblongata*.

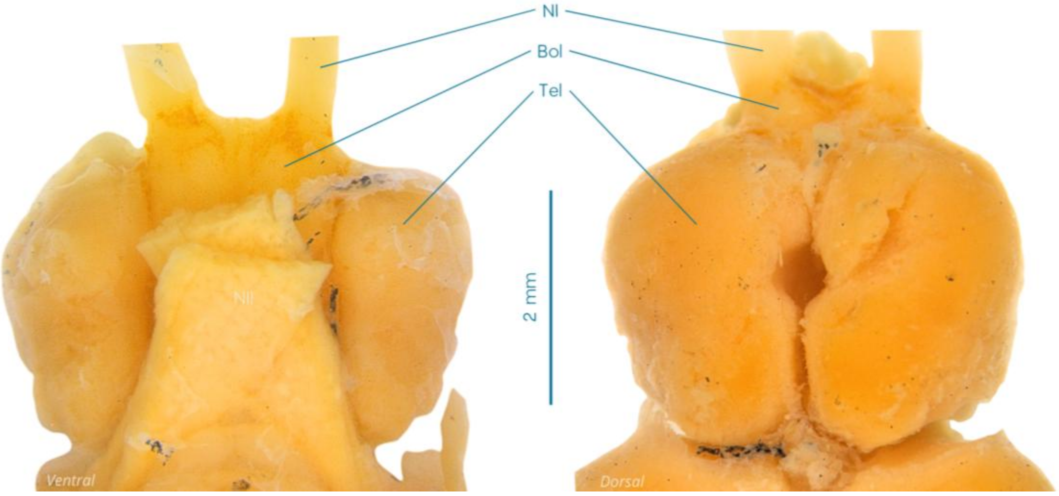


(A): *A. anableps* (B): *A. microlepis*

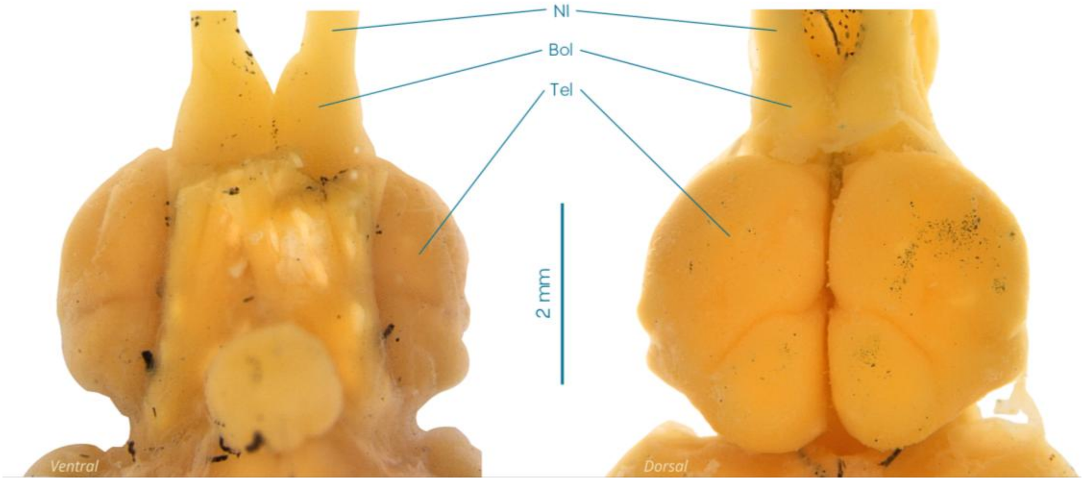
Source: Authors, 2026.

In the *Telencephalon* region, the telencephalon properly exhibits modifications in both shape and size. In *A. anableps*, the telencephalon has a more defined reniform shape and fewer surface irregularities. *A. microlepis*, on the other hand, presents pronounced indentations in the *pallium* and *subpallium*, which provide a notable irregularity to the telencephalic surface (Figure 05). A dorsal indentation is observed, originating at the medial extremity and contouring part of the posterior portion of the *pallium*. The posterodorsal region, distal to the midline, is marked by an indentation that begins at the periphery and vertically contours the entire *telencephalon*, extending into the *subpallium* region. In *A. anableps*, these indentations are not evident, with the exception of the latter, which is visible in dorsal view but poorly distinguishable laterally. Finally, the *telencephalon* of *A. anableps* overlaps the *bulbus olfactorius* to a greater degree, suggesting that the latter is shorter in *A. anableps* and longer in *A. microlepis*. This suggests an enlargement of the *telencephalon* in *A. anableps* (Graphs 01 and 02), which is statistically supported (p = 0.023) and results in the partial overlap of the *bulbus olfactorius*.

Figure 05. Comparison of the dorsal and ventral view of the *Telencephalon* region in the brain across species. Bol: *bulbus olfactorius*; NI: *nervus olfatorius* and Tel: *telencephalon*.



(A): *A. anableps*

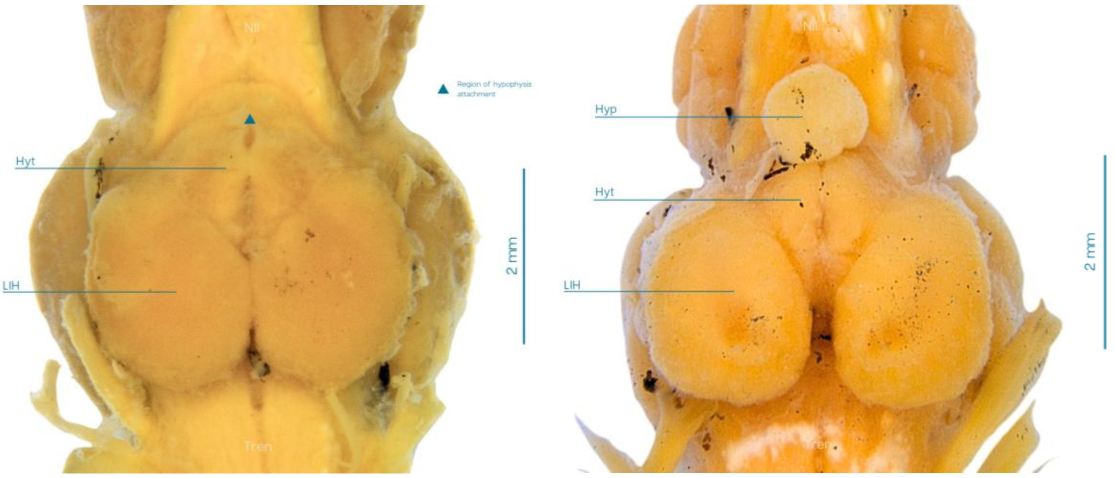


(A): *A. microlepis*

Source: Authors, 2026.

In the *Diencephalon*, the *lobus inferior hypothalami*, a paired structure situated posteriorly to the *hypothalamus*, presents an oval shape in ventral view in *A. anableps*, with predominantly rounded surfaces. In contrast, in *A. microlepis*, the lobes exhibit an approximately trapezoidal shape, with more rectilinear edges (Figure 06). Moreover, semicircular sulci are observed on the ventral surface of the *lobus inferior hypothalami* in *A. microlepis*, which are absent in *A. anableps*.

Figure 06. Comparison of the ventral view of the *Diencephalon* region in the brain across species. Hyp: *Hypophysis*; Hyt: *Hypothalamus*; Lih: *Lobus inferior hypothalami*; NII: *nervus opticus* and Tren: *truncus encephali.*

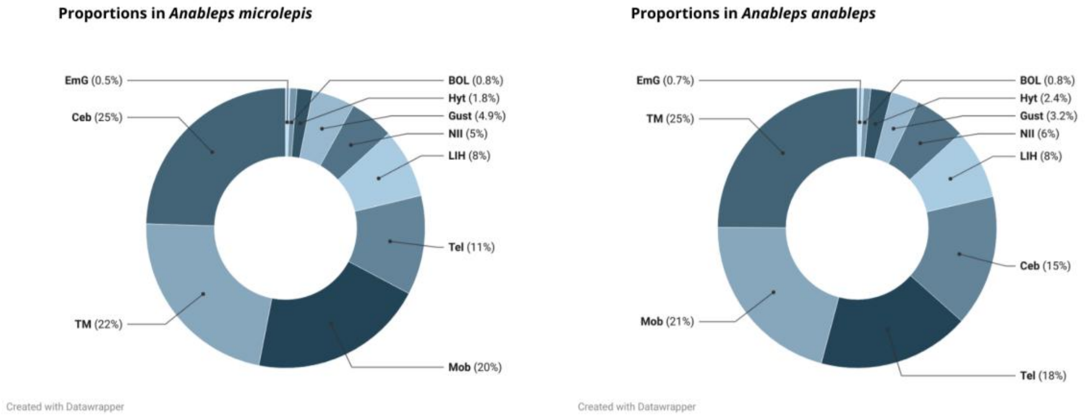


(A): *A. anableps* (B): *A. microlepis*

Source: Author, 2026.

As mentioned, the greatest variations in the relative volume of brain structures between the species are found in the *cerebellum*, *telencephalon*, and *eminentia granularis*. For the remaining structures, the proportions remained similar (Graphs 01 and 02). The *tectum mesencephali*, together with the associated *nervus opticus*, constitutes the most voluminous structure of the *Anableps* brain, occupying on average 27.5 to 31% of the total brain volume.

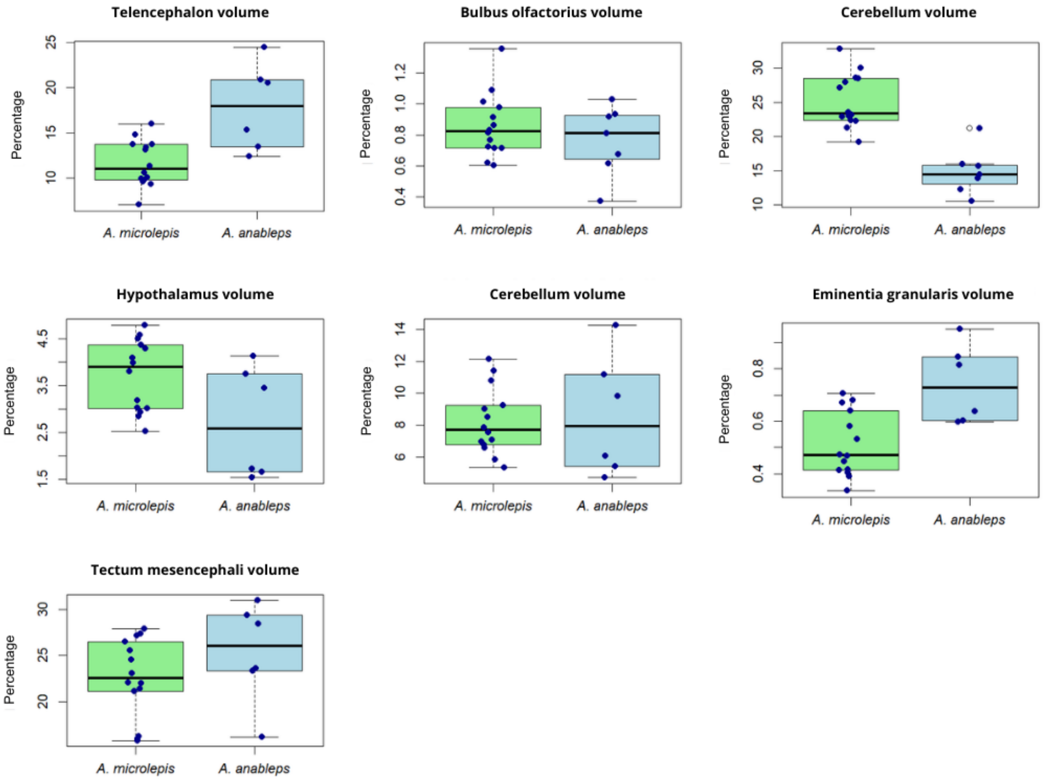
Graph 01. Relative proportions of brain structures.



(A): *A. anableps* (B): *A. microlepis*

Source: Authors, 2026.

Graph 02. Comparison of relative proportions of brain structures between the species *A. anableps* and *A. microlepis*.



Source: Authors, 2026.

Ecology and Behavior

As expected for closely related groups, the species exhibited several shared characteristics. Notable highlights include a larger relative volume occupied by the *tectum mesencephali* and the associated *nervus opticus* (averaging 27.5 to 31%); the width of the *nervus opticus* accounting for, on average, 20 to 25% of the total brain width; a rectangular *cerebellum* with a central constriction overlapping the gustatory lobes; and posterior prominences in the *pallium* and, to a greater extent, in the *subpallium* of the *telencephalon*.

Despite being sympatric species with considerably overlapping niches (Miller, 1979; Nascimento & Assunção, 2008; Watanabe et al., 2014), differences were identified between the brains of *A. microlepis* and *A. anableps*. Studies such as those by Fernandéz et al. (2011) and Abrahão & Shibata (2015) reported similar results when identifying variations in the CNS of congeneric species of *Austrolebias* Costa, 1998 and *Pseudopimelodus* Bleeker, 1858, respectively. The morphometric variations found in the *telencephalon* and *cerebellum* of these *Anableps* species are, therefore, highly informative and may indicate different adaptations developed in response to distinct environmental pressures. According to Korsthal et al. (1998), variations found in the brains of closely related taxa are mostly explained by ecological adaptations, being less affected by phylogenetic relationships. Therefore, ecological-behavioral inferences play a fundamental role here and will be further explored in this section.

One of the characteristics of the *Anableps* brain is the presence of a relatively developed *cerebellum*, which overlaps the gustatory lobes. These lobes and the *eminentia granularis* are less prominent, and in *A. anableps*, the *lobus vagi* is virtually indistinguishable. The *cerebellum* is a cortical structure linked to the functions of balance, motor coordination, and the smooth execution of rapid movements (Butler & Hodos, 2005). Ancestral or benthic fish with sedentary habits tend to possess a reduced *cerebellum*. In contrast, pelagic sharks and fast-swimming teleosts exhibit a more pronounced development of this structure, although such a pattern is not exclusively restricted to these groups (Kotrschal, van Staaden & Huber, 1998). Four-eyed fish are considered difficult to capture by conventional fishing methods due to their high agility and wide visual capacity, which allows them to detect approaching aerial or terrestrial threats from distances of over 10 meters. Their notable ability to perform leaps, whether to escape capture or to reach prey outside the water, reinforces this reputation (Miller, 1979; Turko & Wright, 2015). In addition, schooling behavior, which requires extremely rapid and coordinated movements, is associated with the robustness of the cerebellar structure observed in this group.

The genus *Anableps* performs intertidal migrations as a strategy for better foraging, reducing predator exposure, and exploring more favorable environmental conditions. In this context, Brenner and Krumme (2007) analyzed the migratory pattern of South American four-eyed fish species and identified differences between them. In environments with semi-diurnal tides and high amplitude, *A. microlepis* exhibits a rhythmic and highly predictable migratory behavior, characterized by movement to the intertidal zone during each flood tide and return to the subtidal region during the ebb tide, establishing a regular round-trip cycle with greater displacement. On the other hand, *A. anableps*, in microtidal regions, displays a more opportunistic pattern, remaining adjusted to the tide line. In this case, the advance and retreat of the water's edge determine the movement of the fish, which continuously follows this boundary, exploring newly available resources. Morphometric analyses revealed a higher relative volume of the *cerebellum* in *A. microlepis* and a lower volume in *A. anableps*, which may be related to the distinct migratory patterns between the species. For the migratory pattern of *A. microlepis*, rhythmic and of greater amplitude in environments with strong tidal variation, high coordination

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and motor demand are required. For *A. anableps*, with opportunistic migratory behavior and a relatively more sedentary tendency, the displacement is less extensive and the locomotor effort is lower.

The *tectum mesencephali* is relatively well-developed in *Anableps*, which was expected given its visual specialization associated with the modification of the corresponding sensory organ. The bipartite eyes characteristic of the genus possess an enlarged retina, adapted to capture light stimuli simultaneously from both aerial and aquatic environments (Owens et al., 2011). The *nervus opticus*, intimately related to the retina, conducts the electrical impulses resulting from visual transduction to the brain. Its notable robustness reflects the necessity of concomitantly transmitting signals originating from two distinct environments through a dorsal and ventral retina.

The *telencephalon* of four-eyed fish represents, on average, 11 to 18% of the total brain volume. Considered a higher integration center, the *telencephalon* is directly related to spatial navigation capacity, memory, and the processing of complex environmental information, reflecting more accurately the differences in microhabitat and the spatial complexity of the environment. In contrast, aspects such as diet are more directly associated with primary sensory representation structures, such as the gustatory and olfactory lobes (van Staaden et al., 1995; Butler & Hodos, 2005). Studies with African cichlids have demonstrated variation in *telencephalon* size and its close relationship to the challenges of spatial and environmental complexity (Huber et al., 1997). Fish with a preference for shallower and structurally complex habitats exhibited a more developed *telencephalon*, whereas in shallow and rocky habitats, the gustatory lobes were smaller (van Staaden et al., 1995; Huber et al., 1997).

Fish of the genus *Anableps* inhabit estuaries, dynamic and highly heterogeneous environments (Schettini, 2002). These can be segmented into three different zones: the lower estuary, the outermost region connected to the open sea; the middle estuary, the interaction region between continental and oceanic waters; and the upper estuary, where freshwater predominates but is subject to daily tidal influence (Dionne, 1963 as cited in Schettini, 2002). Middle estuary regions, or mixing zones, are complex and variable, concentrating mangroves and being rich in microhabitats. In this zone, and possibly in transition regions with the upper estuary, populations of *A. anableps* dwell, as they do not tolerate high salinity and are limited to intertidal regions (Nascimento & Assunção, 2008). Conversely, *A. microlepis* tolerates higher salinity levels and can even be sighted in the open sea, thus being characteristic of lower estuary zones (Miller, 1979; Nascimento & Assunção, 2008). This zone is less variable and structurally less complex. With this in mind, such a gradient of structural complexity in the estuarine environment may have influenced the developmental pattern of the *telencephalon* between the species. *A. microlepis*, with a lower relative volume of this structure, is associated with spatially less complex zones, whereas *A. anableps*, with a higher relative volume of the *telencephalon*, is found in microhabitat-rich zones.

CONCLUSION

The results obtained demonstrate that *A. anableps* and *A. microlepis*, closely related species, exhibit significant differences in their brain macroanatomy. These differences appear to be strictly related to the way each species operates within its environment, particularly regarding migratory behavioral patterns and distinct salinity tolerances, which lead the species to occupy different estuarine zones. Thus, a morphofunctional relationship between brain macroanatomy and the behavior/ecological niche of the four-eyed fish is proposed. The foundations of this relationship may be more deeply investigated through the integration of histological and physiological analyses in future studies. In view of the aforementioned relationship, the plasticity of the nervous system is a characteristic that can be observed at the macroanatomical level in fish, as seen in species of the genus *Anableps*. The neuroanatomical divergences found, therefore, illustrate how the CNS acts as a morphofunctional marker sensitive to ecological pressures, reinforcing the importance of neural plasticity as an adaptive mechanism in fish.

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