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THE EFFECT OF HYPOXIA ON BRAIN CELL PROLIFERATION IN WEAKLY ELECTRIC FISH, *PETROCEPHALUS DEGENI*

BY

KAITLIN E. KLOVDAHL

A THESIS SUBMITTED TO

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THE EFFECT OF HYPOXIA ON BRAIN CELL PROLIFERATION IN WEAKLY ELECTRIC FISH, *PETROCEPHALUS DEGENI*

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ABSTRACT

Oxygen levels tend to remain at a steady state concentration in the Earth's atmosphere, yet in some bodies of water, they can fluctuate and decrease drastically. Many organisms that inhabit the swamps, lakes, streams, and parts of the ocean where this occurs have evolved adaptations to manage this environmental uncertainty and continue normal oxygen consumption. The Lwamunda swamp in Uganda is chronically hypoxic, yet it is home to many species, including the electric fish *Petrocephalus degeni*. *P. degeni* are unusual by nature of their immense brain, and the Lwamunda swamp appears ill-suited for maintaining this large, metabolically active organ. To determine the possible mechanisms *P. degeni* employ for survival and brain maintenance in this hypoxic swamp, 33 individuals were collected aiming to analyze their brain cell proliferation. One-third were immediately sacrificed, and two-thirds were transported to a laboratory and divided into hypoxic and normoxic environments for two weeks. All brains were collected, and new brain cell proliferation was quantified using PCNA immunohistochemistry. *P. degeni* from the hypoxic lab condition showed significantly fewer PCNA+ cells than their conspecifics in normoxic water, and individuals harvested directly from the field showed the overall highest density of PCNA+ brain cells. Our results suggest that hypoxia and captivity negatively impacted brain cell growth in *P. degeni*. The activation of hypoxia-inducible factors (HIFs) likely mediated this reduction in brain cell proliferation and the corresponding oxygen demand. Despite showing a reduction in new brain cell growth, *P. degeni* remains capable of surviving and maintaining their large brain in extremely hypoxic conditions.

INTRODUCTION

Life depends on the presence of oxygen. Billions of years ago when oxygen levels increased in the atmosphere, the number of species exploded, diversifying at unprecedented rates (Marshall, 2006). The introduction of oxygen transformed the environment from what was nearly an unhabitable wasteland into an environment that could sustain a multitude of complex life forms. Oxygen constitutes only 20% of the atmosphere, and while the percentage has fluctuated over geological history, it currently hovers near this steady state concentration (Dole, 1965). However, in some bodies of water, the concentration of dissolved oxygen can change drastically within a matter of hours. This variability can apply immense pressure on aquatic organisms, and raises the question, how do they survive in environments that are sometimes hypoxic?

I. Study system: *Petrocephalus degeni* facing hypoxia in a Ugandan lake

Our study organism, the African electric fish, *Petrocephalus degeni,* resides in an environment that is particularly oxygen deficient. Moreover, *P. degeni* has an especially large brain given its overall body mass. Given the high oxygen demands of brain tissue, hypoxic environments appear to be an extremely unsuitable environment for *P. degeni.* For this reason, I examined brain cell proliferation to explore the ways in which they maintain their unusually large, metabolically active brain in a habitat that is hardly conducive to survival.

Petrocephalus degeni is an electric fish species and part of the mormyrid family of fish, endemic to Africa. Mormyrids are commonly referred to as Elephantfish, characterized by their large and flat skull (Sukhum et al., 2016). They exhibit extreme encephalization, and due to the increase in metabolic demand associated with this trait, it may suggest that their brains are less tolerant to changes in oxygen and hypoxia (Sukhum et al., 2016). Chapman and Hulen (2001)

suggested that *P. degeni* inhabiting hypoxic swamps have smaller brains than their conspecifics in fully oxygenated water. The disparity they observed in brain size between *P. degeni* residing in normoxic and hypoxic environments could result from various environmental differences the populations experience, which might include varying oxygen availability, but this is yet to be demonstrated. While this thesis does not investigate the correlation between brain mass and hypoxia, rates of cell proliferation might elucidate mechanisms that *P. degeni* utilize to support their brain in a physiologically challenging environment.

The brains of mormyrids have been recorded to be some of the most metabolically active brains across all ectotherms and endotherms (Chapman & Hulen, 2001). Following trends of mass-based energy expenditure, mormyrid fish should spend the majority of their oxygen consumption on brain maintenance. One species of Mormyridae, *Gnathonemus petersii*, is known to allocate 60% of their oxygen intake for their brain (Chapman & Hulen, 2001). Despite their metabolically needy brain, many mormyrid species demonstrate exceptional tolerance to low oxygen and inhabit water that only contains a fraction of fully-oxygenated water (Chapman & Hulen, 2001).

Populations of *P. degeni* concentrate in and around Lake Victoria, Uganda, its satellite and surrounding lakes, and marshland. In previous decades, the introduction of Nile Perch into Lake Victoria proved disastrous for many native fish species, including *P. degeni*. Fortunately, many of the species that have been decimated by Nile Perch also reside in refugia lakes and swamps that formed thousands of years ago due to the changing tides of Lake Victoria (Chapman et al., 1996). Lake Nabugabo acts as one of these refugia lakes, with the surrounding Lwamunda swamp also host to native fish species, including a population of *P. degeni* (Figure 1a).

The Lwamunda swamp is located between Lake Victoria and Lake Nabugabo, and interestingly, the water in the swamp has considerably less oxygen than both of the lakes. The

concentration of fully oxygenated water typically is around 11 mg L^{-1} , but in the Lwamunda swamp, it hovers around 1.3 mg L^{-1} and can become as low as 0.3 mg L^{-1} near the sediment (Chapman et al., 2002; Kramer, 1987). The lack of flowing water and densely packed vegetation in the swamp likely contributes to the low oxygen content, resulting in a hypoxic environment. The crowded vegetation prevents light from penetrating deeply into the water, therefore photosynthesis occurs at slower rates and oxygen production by plants is hindered. Additionally, the high temperature of the water promotes the decomposition of organic matter, further depleting dissolved oxygen (Chapman, 2015). A multitude of aquatic organisms thrive in the swamp, including *P. degeni* with their immense brains. Studying how *P. degeni* tolerates hypoxia may elucidate ways in which other organisms manage oxygen uncertainty and will further our general understanding of hypoxia responses across species.

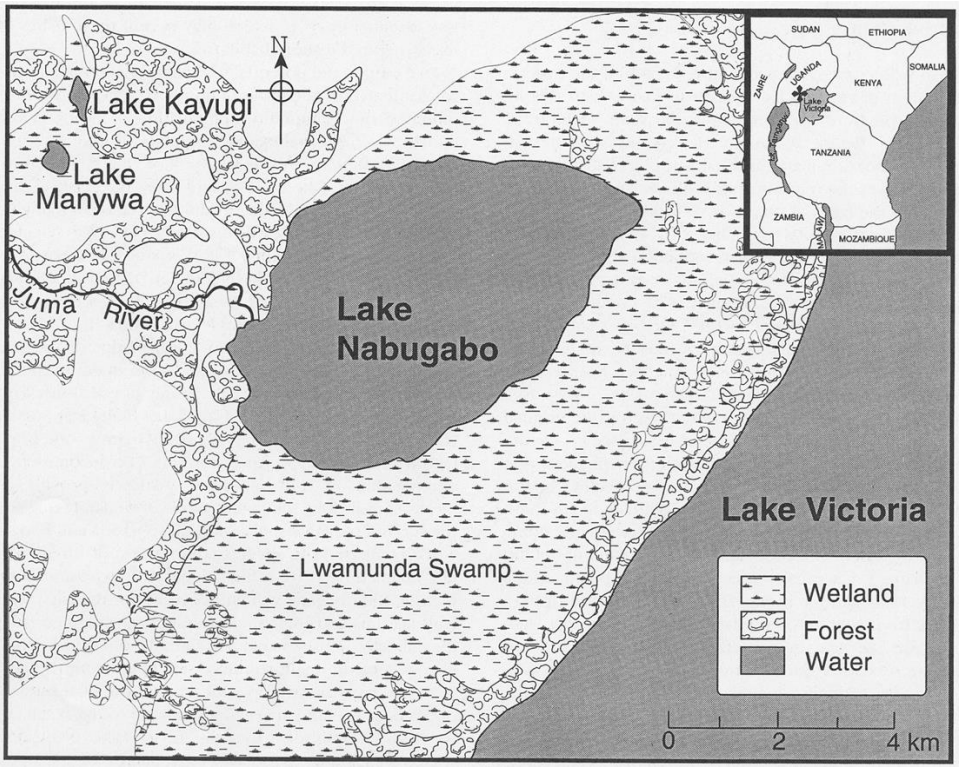

Figure 1a. The location of our study site, the Lwamunda swamp in Uganda (Chapman et al., 1996).

II. Oxygen demand and responses to hypoxia

In hypoxic environments, vertebrates have evolved mechanisms and morphology that allow them to manage this uncertainty and to maintain homeostasis. Without oxygen acting as the final electron acceptor in the electron transport chain, aerobic respiration ceases and sets off a chain reaction that when prolonged, can result in cell death. Without oxygen, sufficient levels of adenosine triphosphate (ATP) cannot be made and cells depolarize, leading to glutamate release and elevated intracellular calcium. As a consequence, apoptosis follows and tissues become damaged as cells are eliminated (Danton & Dietrich, 2005). Tissues differ in their demand for oxygen, and the location of the hypoxic event in an organism's body can have variable and sometimes devastating effects on its function and survival.

The brain is the most metabolically active organ in the human body and utilizes approximately 20% of the oxygen consumed (Rink & Khanna, 2011). Neurons are constantly firing and depleting energy stores, necessitating a high metabolic rate and increased oxygen consumption. Therefore, the brain is highly susceptible to changes in oxygen concentration.

Mammals have been shown to make changes to combat hypoxia at the behavioral level, all the way down to the molecular level. Behavioral changes can manifest themselves as alterations in activity level, while cellular changes are often coordinated by the activation of hypoxia-inducible factors (HIFs) (Majmundar et al., 2010). HIF-1α can promote apoptosis, cell survival, and proliferation pathways depending on available oxygen and redox conditions within cells (Ostrowski & Zhang, 2020). It does so by affecting several cellular processes, such as the initiation of temporary arrest of the cell cycle, a reduction in energy usage, and the secretion of survival and proangiogenic factors (Majmundar et al., 2010). Cellular proliferation and neural stem cell activation can occur through HIF-1α regulation of the Wnt/β-catenin pathway involved in brain

development, and through the upregulation of VEGF production that functions to produce new blood vessels (Carmeliet et al., 1998; Qi et al., 2017). Conversely, under circumstances of prolonged anoxia common in tumor cells, HIF-1α facilitates apoptosis through the stabilization of p53 pathway byproducts, a gene involved in the suppression of cancer cells (Carmeliet et al., 1998; Greijer & van der Wall, 2004). In mammals, activation of hypoxia-inducible factors is one of the most common methods for hypoxia management.

In addition to common adaptations, there are a multitude of other responses that are species-specific. Much research has been conducted on terrestrial species responses to ischemia and hypoxia, but investigations into adaptations in fish are lacking.

III. Adaptations for hypoxia in terrestrial vertebrates

Hypoxia studies have mostly been conducted on terrestrial species which do not commonly face these extreme fluctuations, but rather live in environments where oxygen remains in a relatively steady state. Yet for the species that do experience bouts of hypoxia, their adaptations to this stressor are well researched and understood.

Many burrowing animals that live the majority of their lives underground face chronic hypoxia. Naked mole rats have adapted various ways that they can survive in crowded and suffocating dens. At the molecular level, their hemoglobin has a higher affinity for oxygen than rats that live in open air environments (Larson et al., 2014). Their blood holds onto dissolved oxygen longer, releasing it to their tissues at slower rates and preserving the little oxygen available. Additionally, brain cells from naked mole rats have been shown to resist anoxic depolarization for three times as long as mice, with the cessation of electrical synaptic activity occurring after 13 minutes of anoxia (Larson et al., 2014). This adaptation allows prolonged protection of cells, where

normally they would quickly die. Various adaptations have evolved in burrowing species given that underground living is typically not conducive to air flow and reoxygenation.

Birds are known to be better than most mammals at tolerating low oxygen. With some species flying at heights taller than Mt. Everest or ascending thousands of feet in a matter of hours, they have advanced adaptations to changes blood oxygen levels (Faraci, 1991). To increase oxygen uptake and reduce CO_2, birds are known to hyperventilate. Consequently, the sudden influx of fresh air fills the bronchi in the lungs, and provides the bird more oxygen in an environment where it is not readily available (Faraci, 1991). They also redirect blood flow during hypoxia to tissues that are critical for survival at that time, something mammals are known to do as well (Faraci, 1991). Whether it be underneath the soil to thousands of feet above sea level, oxygen concentrations can decrease from the norm dramatically. The accessibility of the aforementioned species has allowed for meticulous study of their hypoxia adaptations, yet with the majority of aquatic habitats remaining to be explored, organisms with a plethora of hypoxia adaptations may have yet to be discovered. Oxygen concentration fluctuates widely in water, making fish a prime organism for hypoxia studies. Fish continuously face environmental variability, likely necessitating well-developed mechanisms for survival in low oxygen.

IV. Adaptations to hypoxia in fish

Temperature, pH, light availability, salinity, elevation, and anthropogenic forces all influence aquatic oxygen availability and consequently, species survival (Chapman, 2015). Fish share some hypoxia adaptations with mammals, such as blood with high O_2 affinity hemoglobin or the active reduction in metabolic rate; however, they also possess other unique adaptations to manage this strain.

In hypoxic habitats with relatively shallow water, some fish use aquatic surface respiration (ASR) to increase their oxygen consumption (Chapman, 2015). When doing so, they swim to the layer of water just below the surface and allow the oxygen rich water near the surface to flow over their gills. For many species that practice ASR, they only do so when oxygen levels are near their critical oxygen tension, so to avoid increased predation risk and energy expenditure associated with swimming at the surface (Chapman, 2015).

Another way fish increase their oxygen uptake is by enhancing their gas exchange capabilities. Fish species that live in chronically hypoxic environments have larger total gill area surface than their counterparts who reside in normoxic water (Chapman, 2015). Even further, some species change the size of their gills through controlled apoptosis. When the demand for oxygen is high, they actively kill cells between gill lamellae, exposing more of their respiratory organ to the water for increased oxygen absorption (Chapman, 2015). While many adaptations manifest through outward alterations in behavior or morphology, changes can occur on multiple levels of organization, including within the brain.

V. Hypoxia and brain cell proliferation

Hypoxia can influence organisms by modifying the production of new neurons in the brain. The mammalian brain generally stops undergoing neurogenesis after adolescence, with the exception of a few specific brain regions. By adulthood, there is already an established network of brain cells that are responsible for memory, cognition, and overall bodily function. When the brain incurs an injury due to the lack of oxygen, brain cells are often damaged irreversibly, with the function they once served lost. Unfortunately, many mammalian species cannot regenerate brain cells, making the effects of an ischemic stroke especially lasting (Nakatomi et al., 2002). This

suggests that most mammalian species are not well adapted to sudden changes in oxygen availability, but rather have existing brain cells that must learn to manage any fluctuations in order to survive.

Conversely, fish undergo neurogenesis well into adulthood, and can regenerate damaged brain tissue (Zupanc & Zupanc, 2006). While fish and mammalian species have shared adaptations to hypoxia, the piscine brain's ability to form new cells and maintain its plasticity grants fish a tremendous advantage when coping with this stressor. The constant influx of new cells and capacity for neural regeneration make fish a useful subject for the study of neural proliferation, especially under extreme environmental conditions, such as hypoxia.

This phenomenon of brain cell proliferation and regeneration is observed across fish species, even in species like *P. degeni* which reside in water that would appear to be an unsuitable habitat. To better understand the mechanisms behind this distinctive quality in *P. degeni,* I compared fish living in naturally hypoxic waters with those living in lab conditions where we experimentally manipulated oxygen levels. I then compared forebrain cell proliferation in these groups. Any changes in brain cell proliferation after the manipulation of available oxygen may help elucidate the mechanisms behind their tissue maintenance and survival.

The adaptations that allow these fish to exist in a challenging environment while still supporting some of the most metabolically active brains across genera may manifest in a multitude of ways. Does the lack of oxygen decrease overall cell birth in their brains compared to their conspecifics in normoxic water? Or conversely, hypoxic water may increase cell death, to which there are two potential conclusions to be made. A reduced number of new brain cells may be observed, or, brain cell growth may proceed at high rates, a process known as reactive neurogenesis. In mammals, the response to low oxygen is dependent on the ways in which the

brain is damaged by it. Following a stroke, neurogenesis that typically occurs in the sub-ventricular zone (SVZ) switches to the location of the ischemic damage through the migration of SVZ neuroblasts. These neuroblasts differentiate into new neurons that serve as replacements for the ones lost at the site of the damage (Kernie & Parent, 2010). Additionally, after traumatic brain injury, the brain releases various proteins, ions, and growth factors that can regulate neurogenesis and increase cell proliferation at the site of the damage (Kernie & Parent, 2010). Low oxygen may be dealt with in a manner similar to a generalized stress response, or *P. degeni* may treat it as an injury and repair accordingly. In stickleback fish, cortisol levels have been recorded to remain constant even under conditions of induced hypoxia, potentiating the conclusion that fish may have remarkable capabilities for environmental acclimatization (O'Connor et al., 2011). Understanding the ways in which *P. degeni* recognizes low oxygen and the ensuing cellular, physiological, and behavioral adaptations they have evolved may prove instrumental in research concerning brain injury and rehabilitation across species.

MATERIALS & METHODS

(a) Field site and fish capture

Thirty-three *Petrocephalus degeni* were caught in the Lwamunda Swamp (31º50'E and 31º56'30"E to 0º20'S and 0º25'5"S) (Chapman et al., 2002) near Masaka, Uganda. We located them by using an apparatus designed to detect their electric discharges in the water and captured them using a dipnet. Fish were temporarily (~2h) placed into a bucket containing water from the swamp. Eleven fish were sacrificed in the field, and the other two-thirds were transferred to a laboratory for experiments on hypoxia.

(b) Lab experiment on hypoxia

Twenty-two fish were transported to a field laboratory for further experimentation and analysis. All fish were initially placed into water from Lake Nabugabo, in plastic, 50L holding tanks.

Eleven fish were placed into tanks with fully oxygenated water. Air was bubbled into the water using an airstone, which allowed the dissolved oxygen concentration to remain at normoxic levels for the duration of the experiment. After 24 h, one-third of the water volume was replaced, and after 48 hours the full volume was replaced to maintain water quality.

The remaining eleven fish were placed into tanks with hypoxic water. The water was made hypoxic through treatment with sodium sulfite (1.2 g of Na_2SO_3 in 50L of water) and by placing two layers of bubble wrap on the surface to prevent mixing with atmospheric oxygen. Once oxygen levels were < 10%, one quarter of the water was replaced with swamp water every 24 hours. Thus, the oxygen level was always maintained at approximately 10%, except shortly after water replacement, when it increased to 15% for 2 h.

Fish were housed in these conditions for 10 d before they were euthanized and their tissues were collected.

(c) Tissue collection

Fish in the lab were sacrificed immediately after the experiment ended, and the fish that were harvested in the field were euthanized 5-10 minutes after capture, both with anesthetic (0.075%, 2-phenoxyethanol). After body length (within 1mm) and mass (within 0.1g) were measured, the fish were sacrificed by exsanguination, and their blood was collected by syringe. The brain was extracted and weighed, and their electric organs and gills were harvested. The

tissues were placed in a series of solutions for preservation: formaldehyde (4%, 2h) for fixation, PBS (0.1M, 2 x 1h) for rinse and sucrose (25%, overnight) for cryoprotection. The tissues were frozen and stored in the field using liquid nitrogen and kept at -80 ºC in the laboratory.

(d) Immunohistochemistry

To label the proliferating cells, we used the immunohistochemistry protocol for proliferating cell nuclear antigen (PCNA). PCNA is a transcription factor that is expressed during mitosis and serves as the marker for cell birth (Dunlap et al., 2016). Frozen brains were sliced in 30 μm sections and treated with a series of solutions in preparation for the application of the PCNA antibody. The brain sections were first treated with warm HCl (2N, 37ºC, 30 min), followed by borate buffer (0.1 M, pH 8.5, 2 x 10 min), PBS (0.1 M, 1 h), and blocking solution (5% donkey serum, 0.3% Triton X in PBS). The antibody mouse anti-PCNA F-2 (1: 400 in 0.3% Triton X in PBS, Santa Cruz Biotechnology) was applied, followed by PBS (3 x 20 min), and the secondary antibody Cy^{TM}3-conjugated donkey anti-mouse (1:300, Jackson Immunoresearch). All solutions, except for the HCl antigen retrieval step, were done at room temperature.

(e) Cell quantification

The proliferating cells were quantified by counting the PCNA+ cells in two regions of the forebrain: the dorsomedial (Dm) and the ventral (V) telencephalon (Fig. 1b). Using a Nikon E600 epifluorescence scope (200X), the cells were counted bilaterally in a 100 μm band at the medial margin of the forebrain hemispheres. The corresponding area was estimated using NIH imageJ v. 4.0. Cell counts were collected from 2-5 sections per individual fish. The density of proliferating cells (PCNA+ cell mm^{-3}) was estimated by dividing cell counts by the area of each region and

section thickness (30 μm). After cell densities were determined, quantities were compared using the statistical software Prism 3.0.

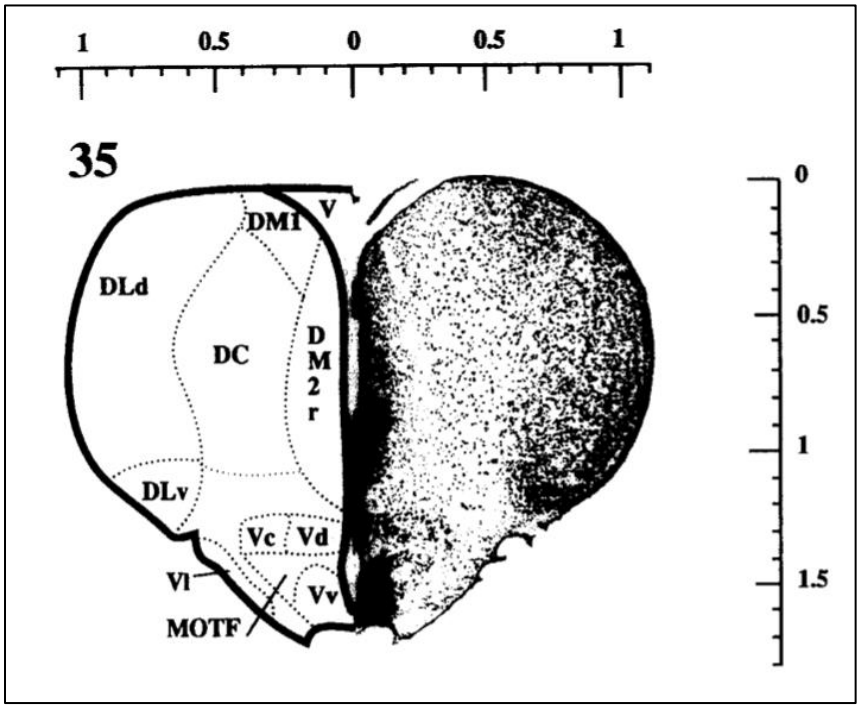

Figure 1b. Coronal cross section of the forebrain of another electric fish species, *Apteronotus leptorhynchus* (Maler et al., 1991), which has the same general structure as the forebrain of *Petrocephalus degeni*. The sections "DM1" and "DM2r" constitute the dorsomedial telencephalon, and sections "Vv" and "Vd" constitute the ventral telencephalon used for cell quantification in *P. degeni* forebrains.

RESULTS

To determine the significance of the PCNA+ labelling patterns observed in *P. degeni,* an ANOVA and Tukey's multiple comparisons statistical test were performed using Prism 3.0 software. The ANOVA test allowed us to determine the variable (treatment, brain region) that most significantly influenced brain cell proliferation among individuals (Table 1). The Tukey's multiple

comparisons test determined the significance of the variation in cell density observed between treatment groups (Table 2).

Table 1. Results from a two-way repeated-measures ANOVA statistical test. The density of PCNA+ cells was influenced by both the experimental treatment (field vs. normoxic captivity vs. hypoxic captivity) and the region of the brain (Dm vs. V).

	DF	**F (DFn, DFd)**	**P value**
Treatment	2	F (2, 20) = 13.810	P = 0.0002
Region	1	F (1, 20) = 5.993	P = 0.0237
Interaction	2	F (2, 20) = 0.275	P = 0.7624

Table 2. Results from a Tukey's multiple comparisons statistical test comparing each treatment group against the others. Significant differences in the quantity of PCNA+ cells found in *P. degeni* brains across treatment groups were observed.

Treatment	**Adjusted P value**
Normoxic vs. Hypoxic	P = 0.0405
Normoxic vs. Field	P = 0.0320
Hypoxic vs. Field	P = 0.0001

In summary, experimental treatment significantly influenced brain cell proliferation in *P. degeni* (F = 13.810, df = 2, P = 0.0002, Table 1). Differences in the quantity of PCNA+ cells in *P. degeni* between the hypoxic test condition, normoxic test condition, and the field resulted directly from exposure to varying levels of dissolved oxygen and their physical environment (field vs. captivity). A greater quantity of PCNA+ cells were observed in the ventral telencephalon (V) than in the dorsomedial telencephalon (Dm) region of the brain (F = 5.998, df = 1, P = 0.0237, Table 1, Fig. 1). The labelling observed in the two brain regions was independent of one another; treatment influenced PCNA labelling in the two brain regions equivalently, since no interactive effect was observed (F = 0.275, df = 2, P = 0.7624, Table 1). Overall, hypoxia and captivity were found to have a significant, negative effect on brain cell proliferation in both regions of the brain that were analyzed.

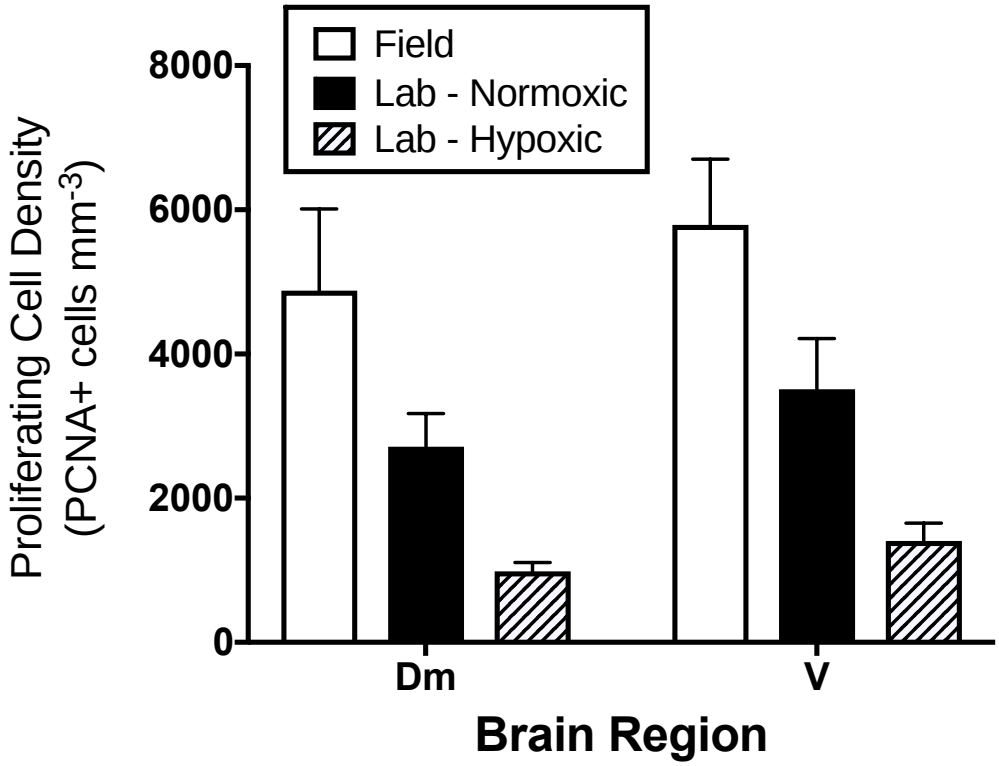

Figure 1. *P. degeni* in the field had the highest density of proliferating cells labelled with the PCNA marker. *P. degeni* in the lab exhibited less proliferation, and those in normoxic condition showed a higher density of PCNA+ cells than those in the hypoxic condition. Cell proliferation was higher in the ventral telencephalon (V) than in the dorsomedial telencephalon (Dm). Error bars indicate standard error.

Brain cell proliferation in *P. degeni* was significantly influenced by their physical environment. *P. degeni* in captivity, in both the experimental hypoxic and normoxic condition, showed reduced quantities of PCNA+ cells compared to conspecifics harvested directly from the field (Fig. 1, Table 2). A significant difference in cell proliferation was observed between individuals in the hypoxic lab condition and those from the field ($P = 0.0001$, Table 2), with fish from the field exhibiting proliferating cell densities 345% greater than those exposed to hypoxia in the lab. Similarly, PCNA+ cell densities were significantly different between individuals in the normoxic lab experiment compared to conspecifics from the field ($P = 0.0320$, Table 2). These

individuals from the field (hypoxic conditions) had density of PCNA+ cells 71% greater than individuals in the normoxic lab condition, despite having less oxygen available to them (Fig. 1). In summary, captivity had a strong, negative impact on brain cell proliferation in *P. degeni*.

The concentration of oxygen in the water significantly impacted brain cell proliferation in *P. degeni*. Individuals placed in the hypoxic lab condition ($[DO_2] < 10\%$) showed the lowest density of PCNA+ cells (Fig. 1). Conspecifics placed into normoxic water in the lab exhibited a density of PCNA+ cells 160% greater than the proliferation observed in *P. degeni* from the hypoxic condition (Fig. 1). This difference in cell density between experimental treatments was significant, resulting directly from the differences in dissolved oxygen concentration in the water ($P = 0.0405$, Table 2). *P. degeni* exhibited increased brain cell proliferation in conditions with high levels of dissolved oxygen than in conditions with reduced oxygen when housed in laboratory conditions.

The overall patterning of PCNA labelling in the forebrain was similar to that of other electric fish, including *Apteronotus leptorhynchus* (shown in Figures 2, 3, & 4) (Because of disruptions due to the COVID-19 pandemic, I was unable to get photographs of *Petrocephalus degeni* forebrains).

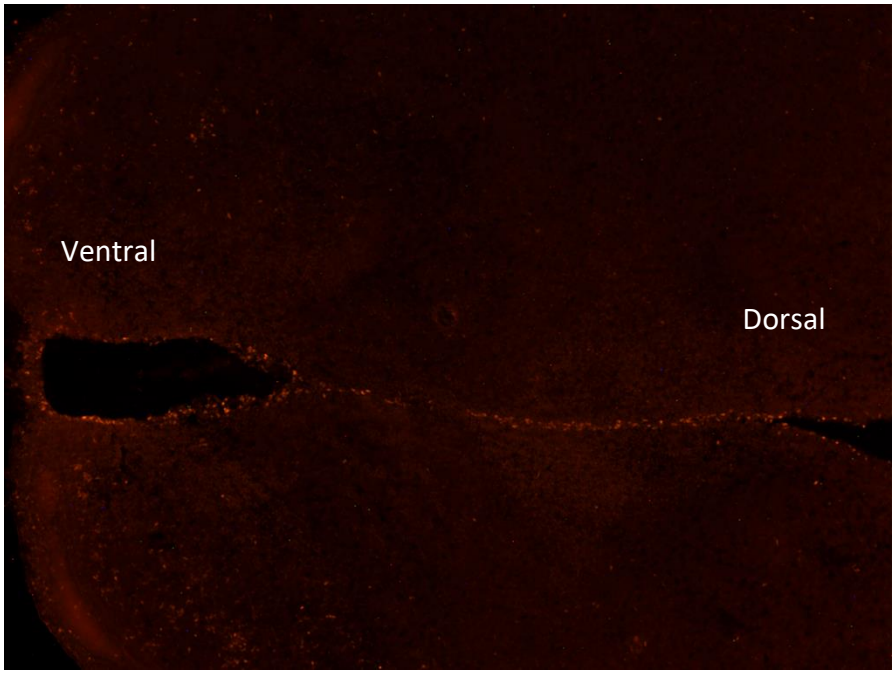

Figure 2. A coronal cross section along the midline of the forebrain in *Apteronotus leptorhynchus* at 10x magnification. The ventral region of the brain is located on the left, and the dorsal region is on the right side of the picture. The fluorescent red points mark new brain cell proliferation, each representative of a PCNA+ cell. This general pattern of PCNA labelling was also present in *P. degeni.*

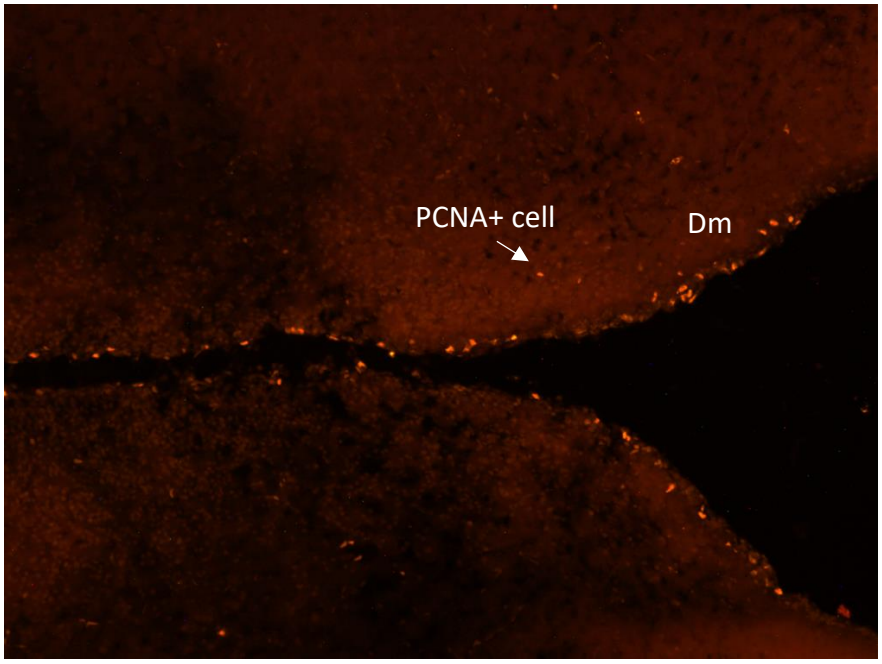

Figure 3. The dorsomedial telencephalon (Dm) region of the forebrain in *A. leptorhynchus* at 20x magnification. Proliferating cells (PCNA+) are labelled with a red, fluorescent marker.

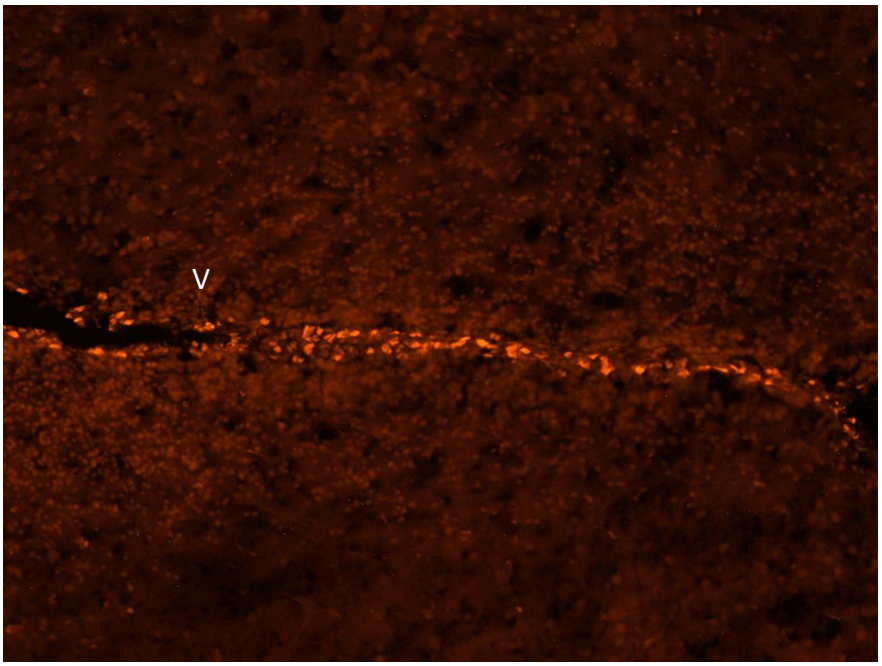

Figure 4. The ventral telencephalon (V) in the forebrain of *A. leptorhynchus* at 20x magnification. Proliferating (PCNA+) cells are labelled with a red, fluorescent marker.

DISCUSSION

I. Hypoxia vs. Normoxia

Petrocephalus degeni exhibited a lower density of proliferating brain cells when housed experimentally in hypoxic conditions than in normoxic conditions (Fig. 1). A dissolved oxygen content of only 10% in the hypoxic treatment significantly reduced the density of PCNA+ cells in both the dorsomedial and ventral telencephalon (Table 2).

In this study, I sought to understand how brain cell proliferation in the remarkably large brains of *P. degeni* is affected by the hypoxic swamp they inhabit. As oxygen is a necessary component in cell homeostasis, growth, and survival, a hypoxic environment is poorly suited to sustain the very metabolically active and abnormally large brain in *P. degeni*. My results showed that the brains of *P. degeni* housed in laboratory conditions responded to hypoxia through a reduction in the quantity of newly proliferating brain cells. Producing new brain cells is an

inherently metabolically costly process, and oxygen may be allocated for protection and maintenance of pre-existing cells rather than new growth. A reduction in oxygen usage in the brain still permits function, and might be necessary to aid in maintaining other cellular processes across the entire individual.

If brain cell proliferation and brain mass are correlated, the observed reduction in proliferation in hypoxia-exposed *P. degeni* might explain the findings of Chapman and Hulen (2001), that fish in hypoxic water had smaller brains than conspecifics in normoxic water. But because changes in brain mass were not examined in this thesis, we can only conclude that the decline in proliferation likely served in conserving oxygen to ensure proper maintenance and function of not only their brain, but the organism as a whole.

Fish species respond to hypoxia in different ways. The duration of hypoxia and its timing during development determines the nature of the organism's response. In early developmental stages, rainbow trout respond to hypoxia by releasing the glucocorticoid hormone, cortisol (Fuzzen et al., 2011). In individuals exposed to hypoxia, an increase in cortisol enhanced expression of the Hsp70 gene that upon activation by stress, aids in neuroprotection. Fuzzen et al. (2011) also found that Hsp70 may aid in stabilizing HIF-1α, the transcription factor that directs a multitude of cellular responses to hypoxia. Sporadic or chronic hypoxia can also be considered as an injury rather than a stress-inducing event. Often during and following a period of ischemia or hypoxia in the brain, HIF transcription factors are activated to offset some of the negative side effects associated with exposure to low levels of oxygen.

The reduction of PCNA+ brain cells in *P. degeni* may minimize the adverse effects of exposure to hypoxia. *P. degeni* might forego producing new cells and instead, like rainbow trout (Fuzzen et al., 2011), activate similar neuroprotective genes to preserve pre-existing cells.

Additionally, the potential activation of hypoxia-inducible factors (HIFs) may explain the minimization of brain cell proliferation in *P. degeni* exposed to hypoxia.

In many species, HIFs are activated when cells are exposed to low levels of oxygen. This complex transcription factor regulates a wide array of cellular responses, ranging from neuroprotective to neurodegenerative (Ostrowski & Zhang, 2020). In *P. degeni,* HIFs may mediate the effect of hypoxia on cell proliferation. Below, I discuss several possible mechanisms by which HIFs might achieve this, specifically through reducing metabolic and growth capabilities of cells, arresting the cell cycle, or facilitating controlled apoptosis (Ostrowski & Zhang, 2020).

1. *Hypoxia and shifts in metabolism*

In low oxygen environments, fish must rely on anerobic metabolism to fuel their physiological processes. Normally, glycolysis supplies energy for cellular growth and survival when oxygen is in unlimited supply. Interruptions in the glycolytic pathway would therefore prevent cells from proliferating. In some species, HIFs can shift cells from using aerobic metabolism and glycolysis, to anaerobic metabolism (Majmundar et al., 2010). This shift away from aerobic metabolism may reduce cell proliferation since enhanced aerobic metabolism has been show to increase neurogenesis in some species, likely through increasing blood flow and neuronal activity (Nokia et al., 2016). One way that HIF-1 α achieves this shift to anaerobic metabolism is by diverting end-products of glycolysis away from mitochondria to suppress oxygen consumption. It does so by repressing the transition of pyruvate into acetyl-CoA, preventing the citric acid cycle from functioning and producing NADH and $FADH_2$ (Majmundar et al., 2010). During exposure to hypoxia, *P. degeni* in our experiment may be forced to decrease their oxygen consumption out of necessity. With extremely low levels of available oxygen, *P. degeni* might

metabolize oxygen sparingly and use anaerobic metabolic pathways to ensure that all cellular processes continue to function, albeit at a depressed rate.

2. *Hypoxia and changes in cell cycling*

HIFs could mediate the reduction in PCNA+ cell density observed in hypoxia-exposed *P. degeni* by slowing down the cell cycling process. Mitotic division is a metabolically costly process (Salazar-Roa & Malumbres, 2017) and, as seen in this experiment, hypoxia might limit mitotic rate. HIF-1α facilitates the arrest in cell cycle at the G_1/S transition by increasing production of inhibitory enzymes and proteins (Goda et al., 2003). It does so by increasing expression of the p27 gene, which prevents hyperphosphorylation of the retinoblastoma (Rb) protein that aids in cell replication. This gene is a kinase inhibitor, and the hypophosphorylation of Rb inhibits progression of the cell cycle at the start of the G_1 phase (Goda et al., 2003). Fewer proliferating cells would reduce oxygen consumption and mitigate some of the potentially harmful effects of hypoxia. In their very metabolically active brains, *P. degeni* may slow the proliferation of new brain cells to avoid the need for increased oxygen consumption in an environment where it is not possible.

3. *Hypoxia mediated apoptosis*

A drastic response to hypoxia is HIF-mediated cell death that typically occurs in organisms exposed to extreme hypoxia or anoxia. In some cases, hypoxia-induced apoptosis stimulates a compensatory increase in cell proliferation, a process called reactive neurogenesis. However, I found in *P. degeni* that hypoxia decreases cell proliferation, so it is unlikely that reactive neurogenesis spurred by apoptosis occurred in these fish. Nevertheless, apoptosis may contribute to separate hypoxia induced changes in their brain.

In other species exposed to low levels of oxygen, HIF's may induce controlled apoptosis (Ostrowski & Zhang, 2020). HIF-induced apoptosis typically occurs through two mechanisms, a) stabilization of the p53 pathway and b) overexpression of proapoptotic pathways (Greijer & van der Wall, 2004). The p53 pathway is activated by environmentally induced stress or cell damage, both of which can occur after exposure to hypoxia to facilitates apoptosis. Under extreme hypoxia, HIFs can be over-activated and increase expression of proapoptotic proteins already present in some cancer cells. The abundance of these proteins, such as BNIP3, inhibits function of the pre-existing antiapoptotic proteins, resulting in intensified apoptosis (Greijer & van der Wall, 2004). Brain cells in *P. degeni* could be undergoing facilitated cell death to reduce potential injury hypoxia incurs on cells. By promoting controlled death, it reduces the potential for detrimental and costly cellular responses to injury to occur in the future. Yet, if apoptosis was the mechanism responsible for the decrease in PCNA+ cell density found in hypoxic *P. degeni*, reactive neurogenesis might also be expected. Reactive neurogenesis can also be aided by the neuroregenerative functions of HIF, such as the upregulation of erythropoietin and vascular endothelial growth factor (Ostrowski & Zhang, 2020). If this mechanism of recovery after exposure to hypoxia was occurring, we might predict an increase in PCNA+ cells in *P. degeni*, the opposite of what was observed.

In *P. degeni*, low oxygen availability likely activated hypoxia-inducible factors (HIFs) or homologous transcription factors in fish that mediated this reduction. *P. degeni* could not proliferate new brain cells at rates comparable to their normoxic counterparts, likely due to the limited supply of, and the need for oxygen elsewhere in the organism. Therefore, *P. degeni*

maintains their unusually large, metabolically active brain in a hypoxic environment through a wide array of cellular and physiological adaptations to maximize their oxygen consumption.

II. Captivity

P. degeni that were held in captivity in both hypoxia ($p = 0.0001$) and normoxia ($p = 0.0320$) had less brain cell proliferation than their conspecifics that were harvested directly from the field (Table 2, Fig. 4). Those in the lab experiments were held in 50L tanks for two weeks, opposed to *P. degeni* taken from the field that never experienced this novel environment.

The negative impacts of captivity have been widely studied and understood across species (Fischer & Romero, 2019). A captive environment will not completely resemble the natural habitat of any species because there are far too many factors to be replicated, biotic and abiotic. The Lwamunda swamp, the natural environment of *P. degeni*, is 4km wide, densely packed with vegetation, home to numerous fish species, and interspersed with streams and lagoons (Chapman et al., 1996, 2002). The multi-faceted and dynamic nature of *P. degeni*'s habitat could not be replicated for the laboratory experiments on hypoxia and normoxia.

Complex sensory stimulation has been shown to have significant effects on brain structure and function of captive animals, and can be achieved through presenting visual, olfactory, and auditory cues that mimic those in the wild (Wells, 2009). With an enriched environment, fish have greater brain cell proliferation (Dunlap et al., 2011; Lindsey et al., 2014), quicker learning and orientation skills, and increased exploratory behavior (Shepherdson, 1994). With only the static, simplified sensory stimulation of captive environments, behavior patterns can stagnate and prevent appropriate reactions to stress or challenge presented in the future (Shepherdson, 1994).

In many mammal and fish species, high levels of the steroid hormone cortisol serve as an indicator of stress. The acute stress response elicited immediately after handling and transport to

captivity typically increases cortisol levels temporarily, but in some cases, cortisol may remain at this elevated level throughout confinement (Fischer & Romero, 2019). The release of cortisol increases the fitness of the organism when facing challenge by facilitating energy investment into behaviors that aid in survival, rather than long-term maintenance (Fischer & Romero, 2019). But when cortisol is chronically high, it may promote adverse effects such as decreased body weight, reproductive capacity, and cognition (Fischer & Romero, 2019). In trout, cortisol has a negative impact on brain cell proliferation, specifically within the telencephalon (Sørensen et al., 2011). Sørensen et al. (2011) administered oral cortisol to rainbow trout for 6d, causing a 50% reduction in new brain cell formation in the telencephalon compared to untreated fish.

The combination of high levels of cortisol and placement into an environment with little stimuli likely contributed to inhibition of brain cell proliferation observed in captive *P. degeni.* Both experimental groups exhibited a reduction in the density of PCNA+ cells compared to the individuals from the field (Table 2, Fig. 4). The stress of capture, handling, and placement into a small, simplified environment likely increased the levels of cortisol in their blood. The relatively short duration of captivity also could have contributed to the reduction in brain cell proliferation observed. Fischer and Romero (2019) found immense variation across species in the time it took for cortisol levels to regulate after initial entry into captivity and upon release into the wild. *P. degeni* probably retained high levels of cortisol for the two-week captive period, and experienced little environmental stimulation, leading to the depressed densities of new brain cells observed in their telencephalon.

III. Dorsomedial telencephalon vs. ventral telencephalon

In *P. degeni* from the field and in both laboratory groups, the density of PCNA+ cells was higher in the ventral than in the dorsomedial telencephalon (Fig. 4). While there was a significant difference in brain cell proliferation between the regions ($p = 0.0237$, Table 2), it likely occurred for reasons inherent to the differences in function in the Dm and V. Exposure to hypoxia was shown to affect both regions equivalently, and an interactive effect between the regions was not demonstrated ($p = 0.7624$, Table 1).

This pattern of brain cell proliferation has also been observed in other fish species as well. Rates of proliferation are higher in the ventral telencephalon than the dorsomedial telencephalon in killifish (*Rivulus hartii* [Dunlap et al., 2019]), South American electric fish (*Apteronotus leptorhynchus* [Dunlap et al., 2017]), and zebrafish (*Danio rerio* ([Grandel et al., 2006]).

Implications & Further Research

Understanding the mechanisms that *P. degeni* employ to survive in extremely hypoxic conditions may help elucidate the ways in which other organisms contend with hypoxic conditions. For species in similar environments where studies on the effects of hypoxia have yet to be completed, the results here may provide insight into adaptive mechanisms. Furthermore, hypoxia is becoming increasingly more common in aquatic habitats due to climate change, forcing more species to cope with this environmental stressor. Studying species frequently exposed to low oxygen may help determine the possibility of similar adaptations adopted by fish inhabiting newly hypoxic water. This research also may apply to humans with brain injury after experiencing a hypoxic-ischemic event, such as a stroke. While a reduction in brain cell proliferation was observed in *P. degeni* here, their abnormally large brain retained enough function for survival.

Knowing the conditions and mechanisms that prompt this form of neuroprotection in *P. degeni* may aid in stroke treatment and recovery in humans.

While this research revealed that hypoxia significantly reduces new brain cell proliferation in *Petrocephalus degeni*, this experiment was limited by the inability to collect specimens from a normoxic field site. Our conclusions regarding the effects of hypoxia are limited to the small population in the laboratory experiments. If *P. degeni* were caught from Lake Nabugabo and compared to their conspecifics from the Lwamunda swamp, it would allow us to determine if hypoxia drives these same effects on brain cell proliferation in wild populations.

As Chapman and Hulen (2001) suggested that *P. degeni* in swamps had smaller brains than their counterparts in normoxic water, studying the correlation between brain cell proliferation and overall brain mass may be of significance. I could only conclude that the reduction observed here likely served to conserve and allocate oxygen for cell longevity and homeostasis of the entire fish, which may indirectly influence brain size. Brain cell proliferation could be directly correlated with increases in brain mass as well. Studying the effects of brain cell *loss* and determining if this drives a decline in brain mass may be beneficial to understanding this potential relationship.

CONCLUSION

The extremely hypoxic water *P. degeni* inhabit does not appear conducive to survival or maintenance of their unusually large brain, suggesting they possess complex adaptations to cope with this stressor. This research examined the effects of hypoxia on brain cell proliferation in *P. degeni* from the Lwamunda swamp in Uganda. Quantified using PCNA immunohistochemistry, *P. degeni* exposed to low levels of oxygen showed a reduction in brain cell proliferation in comparison to conspecifics in fully oxygenated water when both groups were housed in laboratory conditions. Yet, *P. degeni* from the hypoxic field condition exhibited the highest level of brain cell proliferation, demonstrating that captivity had a very significant effect on our results. This necessitates further study of *P. degeni* from normoxic field conditions to determine how hypoxia affects these fish in their normal habitat. For the hypoxic experimental group, fewer PCNA+ cells likely reflect an adaptation to hypoxia that allows *P. degeni* to conserve and allocate oxygen for protection of already existing cells, rather than new proliferation.

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