

Spring 2019

Predators Select for Higher Levels of Brain Cell Proliferation in Trinidadian Killifish, *Rivulus hartii*

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TRINITY COLLEGE

PREDATORS SELECT FOR HIGHER LEVELS OF BRAIN CELL PROLIFERATION IN TRINIDADIAN KILLIFISH,

RIVULUS HARTII

BY

JOSHUA H. CORBO

A THESIS SUBMITTED TO
THE FACULTY OF THE DEPARTMENT OF BIOLOGY
IN CANDIDACY FOR THE BACCALAUREATE DEGREE
WITH HONORS IN BIOLOGY

DEPARTMENT OF BIOLOGY

HARTFORD, CONNECTICUT

6 May 2019

PREDATORS SELECT FOR HIGHER LEVELS OF BRAIN CELL PROLIFERATION IN
TRINIDADIAN KILLIFISH, *RIVULUS HARTII*

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ACKNOWLEDGEMENTS

I would like to express my greatest appreciation to Dr. Kent Dunlap for allowing me to work in his laboratory and being an exceptional mentor to learn from and train under. Aside from academics, Dr. Dunlap has positively influenced my time at Trinity in numerous ways from talking through post-graduation plans to showing me the importance of having lab lunches during the semester. The many biology and life lessons Dr. Dunlap has taught me are things I will remember, for which I hope one day I can teach him a few lessons in return. I sincerely thank Dr. Claire Fournier and Dr. Daniel Blackburn for reviewing several drafts and providing valuable feedback. I must also thank Dr. Matthew Walsh and his team from the University of Texas Arlington for rearing the F1 generation of killifish at his facility.

Margarita Vergara'19 has been a brilliant lab mate and has contributed a great deal of time and thought into this project. I would like to specifically thank her for the number of fish brains she helped me section using the twenty-year-old cryostat in Dr. Dunlap's lab, which took patience and many long nights. Vinnie Salvador has been an invaluable asset during this project because he was responsible for fixing the cryostat when it stopped working properly. I would not have been able to collect data without Vinnie's mechanical skills. I also thank Connor Merinder'19 and Ilan Crawley'19 for assisting in lab procedures during this project. Lastly, I wish to thank the Biology Department for allowing me to conduct biology research during my time as an undergraduate and preparing me in every regard for my future endeavors in the field.

ABSTRACT

Brain cell proliferation is an important form of brain plasticity that has been sparsely studied in natural populations. Killifish, *Rivulus hartii*, from the freshwater streams of Trinidad are a remarkable organism for understanding how brain plasticity is influenced by both internal and environmental factors. Through extensive ecological studies in the region, Trinidadian killifish have been used to determine how predation directly effects brain cell proliferation and brain size. In wild populations, waterfalls in the streams block predator movements upstream, thereby creating distinct populations of killifish – i.e., killifish living with abundant predators (high predators, HP) and killifish living with no predators (*Rivulus*-only, RO). In the present study, fish were caught in HP and RO locations from three replicate streams at a total of six populations. In a common garden study, the F1 population from the same RO and HP streams were reared in captivity under the same living conditions. Immunocytochemistry for proliferating cell nuclear antigen (PCNA) was performed on brains from both wild and common garden killifish to quantify the amount of cell proliferation in the forebrain and midbrain. In the wild, killifish from HP streams had significantly more cell proliferation throughout the brain indicating that predator presence enhances cell proliferation non-specifically. In captivity, fish derived from HP streams also had increased cell proliferation, showing that the population differences in the wild are likely due to intrinsic, evolved genetic differences among populations. This combined study suggests that predation has caused brain cell dynamics in RO and HP killifish to differ genetically, since the results from the common garden experiment paralleled the findings from the field study.

INTRODUCTION

The environment and the vertebrate brain have an intimate, yet complex relationship. In a multitude of ways and combinations, environmental factors can elicit either positive or negative changes to the brain. Based on previous studies, it is apparent that ecological factors, brain cell proliferation, and brain size are interrelated (Gonda *et al.*, 2013; Dunlap *et al.*, 2016, Walsh *et al.*, 2016). The complexity of this relationship with the environment suggests a cellular basis for brain size variation (Fig 1).

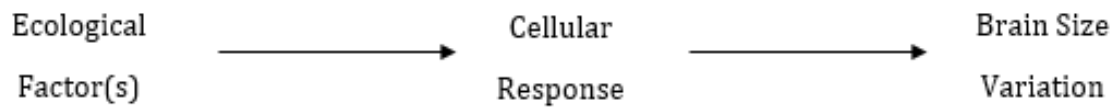


Figure 1: A simplified pathway of how environmental factors influence brain size through cellular processes. Ecological factors elicit changes in cellular dynamics and cell composition in the brain which may ultimately change the morphology of the brain or specific regions of the brain.

However, the exact mechanism underlying this relationship is unknown. Understanding the cellular processes contributing to brain structure helps explain the range of morphological responses a brain has to the environment. For instance, environmental factors are known to alter the cellular composition of the brain by influencing: rate of cell proliferation, rate of cell death, the differentiation of neural stem cells, and survival rate of newly born cells (Dunlap, 2016; Soutsckek and Zupanc, 1995; Soutsckek and Zupanc, 1996; Zupanc et al., 1998). These cellular processes may be largely responsible for ecological and evolutionary changes in brain morphology. Knowing that the environment significantly influences the cellular and morphological structure of the brain, I examined

how one component of the environment, predators, affects cell proliferation in the brains of Trinidadian killifish, *Rivulus hartii*.

To study the effect of predation on cell proliferation, I examined fish from six populations of Trinidadian killifish in three independent streams. Each stream has an upstream population of killifish that lives without predators (*Rivulus*-only, RO) and a downstream population that lives with a high abundance of predators (High Predation, HP), or with a low abundance of predators (Low Predation, LP). Because of this natural occurring separation, I could observe the effects of predator presence or absence on brain cell proliferation.

One important question in examining population differences in brain cell proliferation and brain size is whether the differences are phenotypic responses to predation or intrinsic differences that manifest trans-generationally. By comparing wild-caught population and populations reared in a laboratory common garden setting, I sought to determine whether these differences are attributable to a phenotypic plasticity within a generation or natural selection across generations.

Understanding neurogenesis within context of brain growth and allometry

Animals grow at different rates for different periods and to different sizes (Kotrschal *et al.*, 2012). Fish are more flexible and dynamic in their physiology and adult morphology compared to mammals and birds, mostly because of their unique growth and life history patterns (Dunlap, 2016). They are especially sensitive to environmental factors because most fish are ectotherms, have a great capacity for regenerating portions of their body following injury, and ability to change sex in some species (Dunlap, 2016). The brain is a

plastic organ which makes it susceptible to changes in external conditions. The growth of the brain is especially interesting to biologists because of its interaction with the environment (Gonda *et al.*, 2013). A fish's brain grows allometrically (disproportionately) with body size (Kotrschal *et al.*, 2012). Moreover, most fish species display indeterminate growth, in contrast to birds and mammals that have determinate growth (Dunlap, 2016). Most traits vary to some degree among populations, but because of these attributes, fish populations may display an especially large variation in brain size because of these environmentally influenced growth patterns.

The complexity of the environment and its equally complex influence on the brain makes brain development highly responsive to environmental factors (Dunlap *et al.*, 2011). A species' growth pattern is affected by availability of food, presence of predation or competition, and the physical environment (Schulte, 2014). Therefore, species evolve different growth strategies in response to these ecological pressures (Kotrschal *et al.*, 2012). Evolutionary changes in brain size occur within the context of these larger changes in body growth and life history. These differences in brain size and structure suggest a complex relationship between brain growth and ecological factors that has led to enormous variation in brain development (Kotrschal *et al.*, 2012), and it is unclear whether all external factors affect the brain in the same fashion.

A significant and under studied process in the brain is cell proliferation. Fish are a useful model for studying brain cell proliferation due to their naturally high rate of cell proliferation during adulthood (Dunlap, 2016). For example, the electric fish species, *Brachyhypopomus gauderio*, has cell proliferation rates that vary by 25-fold depending

on their physical and social environment (Dunlap, 2016). Fish as a group have especially high rates of cell proliferation. The high level of brain cell proliferation paired with a naturally high degree of responsiveness to environmental changes suggests that cell proliferation is a prominent process in brain plasticity, which may serve as an important influence on behavioral responses to the environment (Dunlap, 2016).

Sources of variation for population differences in brain: phenotypic vs. genetic

Most studies on the effect of predation on fish brains have compared fish from natural populations exposed to differing levels of predation. Such population differences could arise in two ways: individuals responding within their lifetime to the presence of predators – a form of phenotypic plasticity – or predators selecting against certain genotypes and thereby causing transgenerational effects on the genetic control of brain development.

The environment consistently elicits change, and this occurs in all domains of life. For example, bacteria can switch from glucose to lactose metabolism depending on which sugar is more abundant in its environment (Deutsche *et al.*, 2006). From living in extreme habitat conditions, changing aerobic conditions will alter the growth rate of archaea (López-García and Forterre, 2008). An Arctic fox expresses a brown or white coat depending on the season (Underwood and Reynolds, 1980) (Våge *et al.*, 2005). In these examples, environmental pressures typically cause phenotypic changes. In fact, Fusco and Minelli (2010) theorized that an individual's trajectory is the result of the unique interaction between its genome(s), and the temporal sequence of external pressures it is

exposed to during its life (Fig 2). Therefore, I will distinguish between the two common forms of change – phenotypic plasticity and genetic divergence.

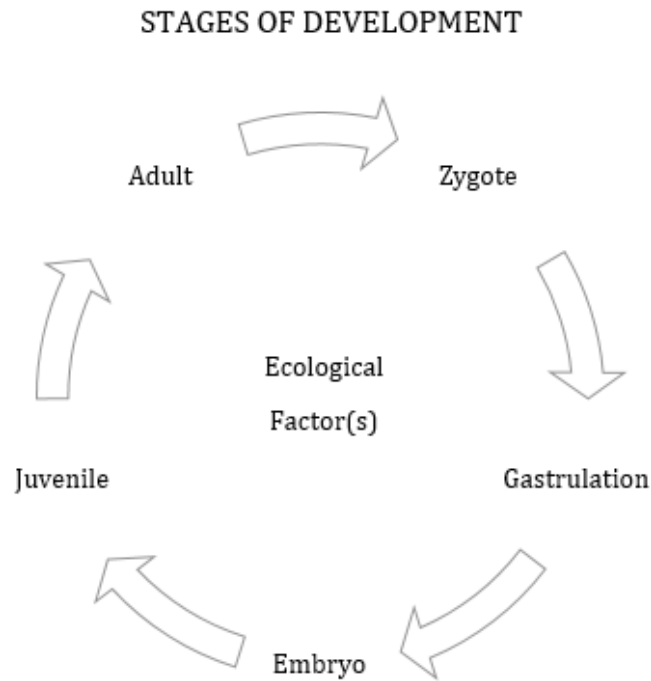


Figure 2: The external environment is capable of influencing the development of an organism during all stages of life. Significant changes can occur depending on the degree, temporal, and spatial appearance of ecological factors. Such changes may result in a phenotypic response to ecological factors or cause a genetic change in the population – natural selection.

Phenotypic plasticity is the ability of individuals to produce different phenotypes when exposed to different environmental conditions (Pigliucci *et al.*, 2006). This can be a response to predation, intraspecific competition, or food availability (Schulte, 2014). The responses vary depending on the time of exposure (Fusco and Minelli, 2010). If a human child (2 y/o) experiences malnutrition for an extended period, it will most likely be shorter and have cognitive disabilities compared to another individual that was fed an adequate diet during development (Winick and Rosso, 1969). What makes these

responses phenotypic is that changes to an individual's phenotype do not affect its genes; thus, its offspring are not affected.

Responses to external pressures that are heritable and cause a change in gene frequency within a population illustrate the second type of response to the environment – genetic divergence. Such changes in populations may come about through natural selection on individuals of a populations. Individuals must first experience the negative or positive effects of the environment and those that best survive and reproduce pass their genes on to the next generation, leading to adaptive evolutionary change within the population. However, there is no general agreement on whether plasticity can promote evolutionary change in populations, or whether it can accelerate or slow genetic divergences (Fusco and Minelli, 2010). In my study, this will be a major question and determining what type of response external pressures are eliciting.

Predator effects on brain size

Brain size is strongly associated with a large variety of fitness-related cognitive and sensory abilities (Samuk *et al.*, 2018). Relative brain size is thought to be determined by a balance between energetic costs of brain tissue and the benefits of enhanced cognitive and sensory abilities (Samuk *et al.*, 2018). Predators have been shown to influence brain size in fish in a number of studies (Kotrschal *et al.*, 2015; Walsh *et al.*, 2016; Samuk *et al.*, 2018; Redon *et al.*, 2018). Moreover, increased brain size is associated with increased cognitive ability, shifts in personality, and increased anti-predator behavior (Walsh *et al.*, 2016). It has been theorized that fish in predator dense environments require larger brains to learn and adapt faster to threats (Gonda *et al.*, 2013). In summary, these studies

suggest that a larger brain is a smarter brain. Based on the simplified diagram (Fig 1), it is assumed that enhanced cellular processes, such as cell growth, promote a more reactive and flexible brain.

In two different studies, Kotrschal *et al.* (2014; 2015) found that guppies, *Poecilia reticulata*, that were selected for larger brains had increased cognitive abilities and survival. Large-brained males were almost twice as fast at finding a mate as small-brained males (Kotrschal *et al.*, 2014). In a later study (2015), Kotrschal *et al.* found that large-brained females had a 13.5% increased survival compared to small-brained females. Interestingly, brain size had no effect on male survival. Furthermore, male Trinidadian guppies had heavier brains relative to their body size in response to predation (Reddon *et al.*, 2018). In fact, this observation was found in the wild-caught and lab reared guppies, which showed that predation selected for these traits (Reddon *et al.*, 2018). This was one of few studies that have observed a transgenerational effect of predation on brain size in fish.

In contrast with these studies, other recent studies have revealed that smaller brains may be selected in predator environments because brain tissue has a high metabolic cost. Faced with a high predation threat, fish might benefit from allocating this energy to increase body size and strength rather than brain growth (Samuk *et al.*, 2018). After two generations of selection, Samuk *et al.* (2018) found that sticklebacks from the predator exposed treatments had significantly smaller brains than fish without predator exposure. In another study, male killifish from high-predator localities had significantly smaller brain sizes compared to males from predator-free populations (Walsh *et al.*,

2016). This study showed the apparent influence of predators on brain size, but the underlying cellular processes in the brains of these killifish affected by predators is unknown. In my thesis, I examine males from HP and RO populations to assess if there are differences in brain cell proliferation between these two predator environments. Since environmental factors influence brain size, I ask: what cellular processes underlie environmentally induced changes in brain size?

Predator effects on brain cell proliferation

Despite the shortage of studies, cell proliferation is a major process that occurs in the brain, and brain cell proliferation rate is highly reactive to environmental pressures. Brain cell proliferation in the forebrain of a Panamanian electric fish (*B. occidentalis*) is lower in fish from high predator streams than those from low predator streams (Dunlap *et al.*, 2016). These fish were exposed to natural variation in predator abundance and to predator-induced tail injury. In both cases, forebrain cell proliferation was lower in fish facing greater predator exposure (Dunlap *et al.*, 2016).

This field study (Dunlap *et al.*, 2016) described only correlations between predator exposure and brain cell proliferation. Dunlap *et al.* (2017) sought to test this correlation by experimentally exposing fish, *B. gauderio* and *Apteronotus leptorhynchus*, to predator stimuli. Predator stimulus was created by either a tail amputation or repeated chasing of an intact (non-amputated) fish during brief periods of the day for seven days (Dunlap *et al.*, 2017). This experiment found that fish experimentally exposed to either form of predator stimulus had decreased brain cell proliferation in the telencephalon (Dunlap *et al.*, 2017). These results demonstrate phenotypic plasticity in

brain cell proliferation and confirm that predator exposure causes changes in brain dynamics. An important point here is that the experiment shows that predators cause changes, while the field study only shows that predators occur with (correlate with) low proliferation.

Though this investigation on Panamanian electric fish (Dunlap *et al.*, 2016) provides insights on the effects of predation on cell proliferation, there are some limitations to this study: (1) populations, even with drainages, were geographically far apart, so population differences in cell proliferation could be due to environmental variables other than predator density. (2) Electric fishes cannot be bred in a lab setting; hence one cannot assess intrinsic differences between populations. (3) Predator abundance varied quantitatively across the populations rather than presence/absence. The study did not answer whether these neurogenic changes are strictly environmentally induced (natural predation) as a behavioral response or if there is a genetic component.

Trinidadian killifish as a model organism

Two features of Trinidadian killifish make them a particularly good model for examining how the environment influences the brain. First, killifish populations are known to differ both genetically and phenotypically (Schulte, 2014). Furthermore, the ecological factors in this region have been extensively studied. Killifish populations are located in close proximity across a series of streams that span the Northern Mountain Range of Trinidad. In this terrain, waterfall barriers have isolated a number of fish populations. Thus, many streams contain populations of killifish that are living with predators (HP and LP) and other populations without predators (RO). Walsh and Reznick (2009) evaluated the

physical variables among killifish populations, and found no significant differences between the physical environments. The oxygen saturation, temperature, salinity, and pH of the water as well as the stream width and depth were essentially the same in each of the streams and various killifish populations– HP and RO (Walsh and Reznick, 2009). Consequently, the Trinidadian killifish is an excellent model to examine the traits influenced by predation (Schulte, 2014). These ecological factors enable a “3x2 Design” (three streams contain two killifish populations that differ in predation pressure) and any population differences within a stream can be directly attributed to predation.

My thesis consists of two parts: (a) a field study of wild caught killifish and (b) a common garden laboratory experiment using the same wild caught populations. Wild caught killifish were first studied to see how brain cell proliferation correlates with predation and body size. The purpose of conducting the common garden experiment was to address whether population differences are attributable to phenotypic plasticity or genetic differentiation– i.e., an evolutionary explanation (Reddon *et al.*, 2018). If population differences in the common garden reared killifish mirrored the wild caught data, then this would indicate that the killifish population genetically diverged because of selection by predation. Population differences would be attributed to a predator elicited phenotypic response if the common garden experiment showed that there were no differences between the HP and RO populations.

I found that killifish populations from high predation environments had significantly more brain cell proliferation than in environments containing no predators, or low predation. This population difference observed in the field was also found in lab

reared fish from common garden experiment. Hence, these recent results regarding brain cell proliferation suggest predation has genetically influenced Trinidadian killifish to evolve significantly more brain cells and smaller brains (Walsh *et al.*, 2016).

MATERIALS AND METHODS

(a) Wild-caught killifish population sites and capture (Walsh et al, 2016)

Adult killifish (n= 7-9 per site; total body length 45-82mm; mean = 59.1 ± 1.5 mm) were captured via dip net from three independent rivers – Aripo, Arima, and Yarra – in the Northern Mountain Chain of Trinidad during August 2017 (Fig 3). Only males were collected because Walsh et al. (2016) showed that predation affected the brain size of male killifish. For each river, there were two sample locations – a high predation (HP) site that was abundant with piscivorous predators, pike cichlids (*Crenicichla alta*) and wolf fish (*Hoplias malabaricus*), and another site that had no predators (*Rivulus*-only, RO). The Yarra River had a low predation site (LP) rather than a RO locality. Six capture sites were identified to allow for a comparison of killifish from HP sites and killifish from LP or RO sites. Fish were sacrificed on site and the brains were kept frozen with dry ice prior to being stored in laboratory conditions.

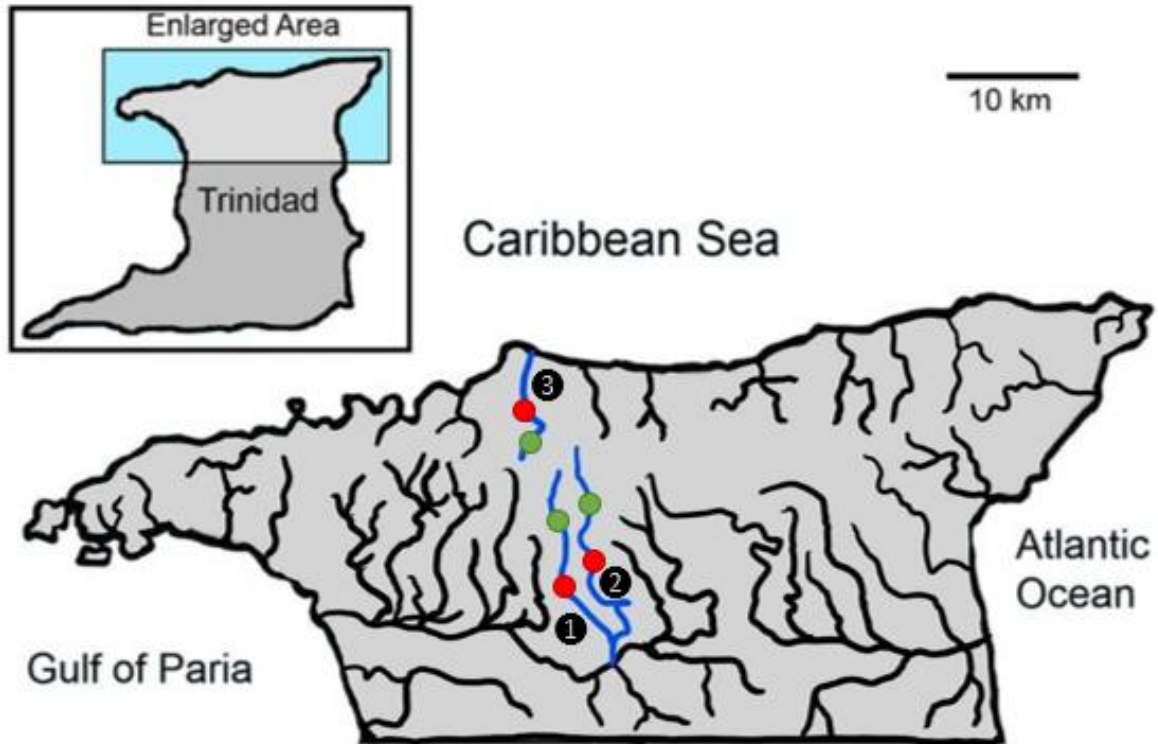


Figure 3: River map of the Northern Mountain Range of Trinidad after Zandonà et al., 2017. The rivers highlighted in blue represent the (1) Arima R., (2) Aripo R., and (3) Yarra R. Red dots represent HP collection sites and green dots represent RO localities.

(b) F1 captive reared killifish (Walsh *et al.*, 2016)

For the common garden experiment, wild-caught killifish were collected from RO and HP population from the Aripo and Arima rivers and each distinct population was maintained in uniform laboratory environments at the University of Texas, Arlington from Jan-Jun 2018. The first generation of common garden reared fish were generated by placing 10 wild-caught females individually in 9 liter aquaria supplied with artificial spawning substrate and randomly paired with a male from the same locality. (E.g., Arima HP female x Arima HP male). Eggs from these pairings were incubated in Petri dishes for a controlled growth. The newly hatched larvae from each pairing were then reared in aquaria, with 8-10 fish per aquarium, and fed a liver paste and larval *Artemia ad libitum*.

Procedures and methodology on laboratory breeding and husbandry were reported previously (Walsh and Reznick, 2008). Fish were sacrificed at 54-82 d post hatching (total body length= 27-37, mean = 32.1 ± 0.3 mm). The brains of fish (n= 5-9 per population) were processed and frozen for cell proliferation assays.

(c) Tissue collection in the field and lab

Brain dissection and processing procedure were similar to that reported previously (Dunlap *et al.*, 2016). Fish in each population were euthanized 5-10 minutes after capture with anesthetic (0.075% 2-phenoxyethanol). Once body length (mm) was recorded, brains were dissected and placed in a series of solutions on ice: formaldehyde (4%, 2h) for fixation, PBS (0.1 M, 2 × 1h) for rinse and sucrose (25%, overnight) for cryoprotection. Brains were then frozen with pulverized dry ice the next morning. Brains were stored on dry ice in the field and afterwards at -80°C in the laboratory.

(d) Immunohistochemistry

To label proliferating brain cells, I used immunohistochemistry for proliferating cell nuclear antigen (PCNA), a transcription factor in mitosis and a marker for cell birth. I used a procedure similar to that reported previously (Dunlap *et al.*, 2016). Brain sections (30 µm) were treated sequentially with HCl (2N, 37°C, 30 min), borate buffer (0.1 M, pH 8.5, 2 × 10 min), PBS (0.1 M, 1 h), blocking solution (5% donkey serum, 0.3% Triton X in PBS), mouse anti-PCNA F-2 (1 : 400 in blocking solution, Santa Cruz Biotechnology, sc-25280), PBS (3 × 20 min) and CyTM3-conjugated donkey anti-mouse secondary antibody (1 : 300, Jackson Research Laboratories). All solutions (except HCl antigen retrieval step) were done at room temperature.

The abundance of proliferating cells was quantified by counting unilaterally the PCNA+ cells in three forebrain regions – dorsolateral (DL), dorsomedial (DM) and ventral (V) – and midbrain regions in the periventricular zone (PVZ) (Fig 4). These brain sections were equivalent to axial regions 30-36 (forebrain) and 14-19 midbrain as described in the brain atlas of electric fish (Maler, 1991) In each region, PCNA+ cells were counted blind in a 100µm band at the periphery of the brain or ventricle using a Nikon E600 epifluorescence scope at 200X, and the corresponding area was estimated using NIH ImageJ v. 4.0. The density (PCNA+ cell mm⁻³) was calculated by dividing cell counts by the area of each region and section thickness (30 µm).

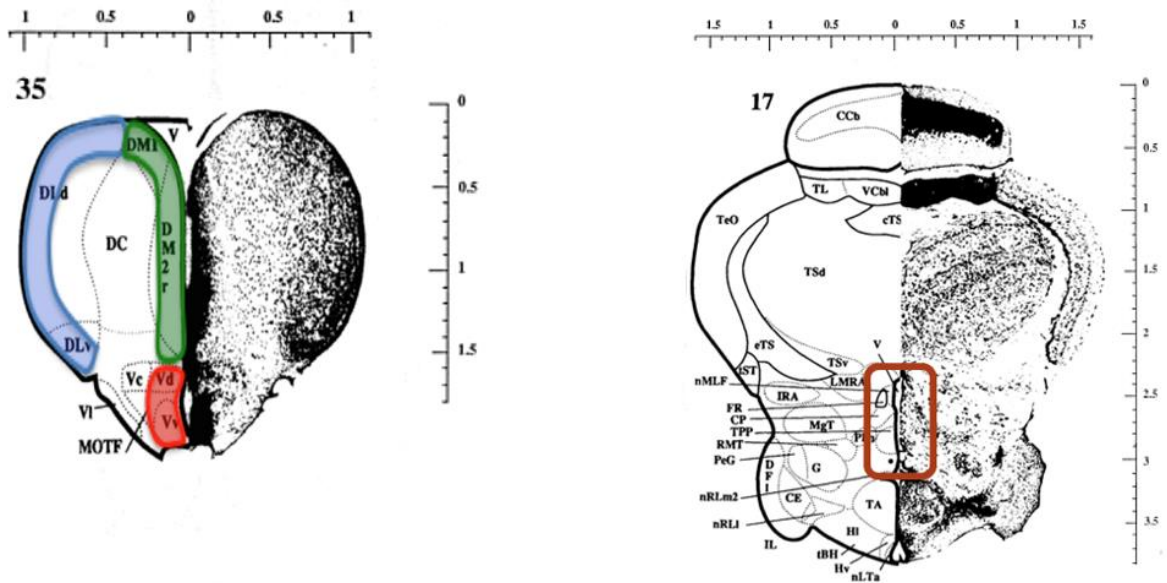


Figure 4: Brain cross-section of the forebrain section 35 and midbrain section 17 from Maler fish brain atlas (1991). [Left] Forebrain with labeled regions: blue = dorsolateral (DL), green = dorsomedial (DM), and red = ventral telencephalon (V). [Right] Midbrain with periventricular zone (PVZ) enclosed by red box.

(e) Statistics

For both wild-caught populations and the captive common garden experiment, I used a three-way ANOVA with stream, predation environment, and brain region as the independent variables, and density of PCNA⁺ cells as the dependent variable. Values are expressed as mean plus/minus standard error. Statistical analyses were conducted in GraphPad Prism 7.0.

RESULTS

Field Study. Fish living with abundant predators (HP localities) had significantly greater density of PCNA⁺ cells than fish living in RO/LP localities (d.f. = 1, F = 101.51, P = <0.0001; Table 1, 2, and Fig 5). Moreover, the three forebrain regions and the midbrain PVZ were all statistically different from one another (d.f = 3, F = 16.56, P = <0.0001; Table 1, 2, and Fig 5). Brain cross-section images (Fig 6a-d) show an abundance of PCNA⁺ cells in the HP fish and a lack of PCNA⁺ in the RO fish (Fig 6e-h). The three-way ANOVA confirmed that the streams in the Trinidad Range are replicates; that is, there were no significant effects of stream and no interaction between stream, brain region, or predator regime.

Table 1. ANOVA results showing the effect of predation environment on the density of proliferating cells in four brain regions of wild-caught fish.

effect	d.f.	<i>F</i>	<i>P</i>
brain region	3	16.56	<0.0001
stream	2	0.75	>0.05
predation	1	101.51	<0.0001
brain region X stream	3	0.21	>0.05
brain region X predation	3	1.99	>0.05
stream X predation	2	0.01	>0.05
Brain region X stream X predation	3	1.89	>0.05

Table 2: Mean density (\pm SE) of proliferating cells (PCNA+ cell/mm³) in four brain regions in wild-caught killifish collected from sites with differing predation pressure.

Stream	Predation	N	Forebrain			Midbrain
			DL	DM	V	PVZ
Arima	RO	8	18113 \pm 2107	14793 \pm 2878	23725 \pm 5490	9400 \pm 1024
	HP	8	36918 \pm 3553	40261 \pm 6810	39128 \pm 4581	22133 \pm 3501
Aripo	RO	8	13947 \pm 2521	16167 \pm 2382	19260 \pm 5417	10087 \pm 1956
	HP	7	37512 \pm 3510	31338 \pm 3322	45123 \pm 4431	18504 \pm 2315
Yarra	LP	9	21464 \pm 825	21177 \pm 1412	22962 \pm 2438	14531 \pm 2015
	HP	9	32819 \pm 2724	36005 \pm 4735	38164 \pm 3489	26058 \pm 2848

RO = *Rivulus*-only (no predators); HP = High predator density; LP = low predator density, DL= dorsolateral telencephalon, DM= dorsomedial telencephalon, V = ventral telencephalon, PVZ = periventricular zone

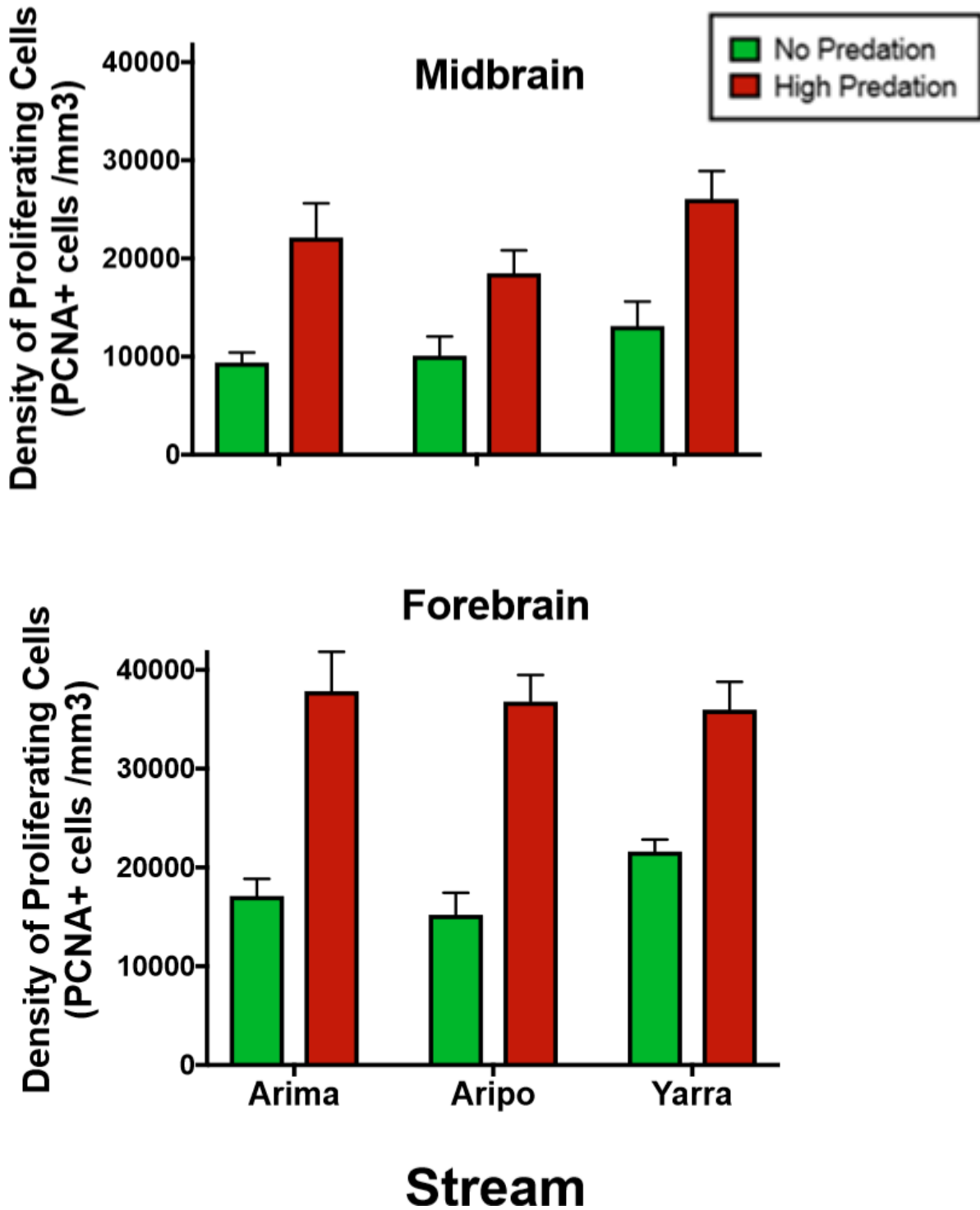


Figure 5. The density of cell proliferation in the forebrain and midbrain of wild-caught killifish from HP and RO/LP populations in the Arima, Aripo, and Yarra River. In this figure, forebrain regions were pooled as there was no significant predator x brain region interaction.

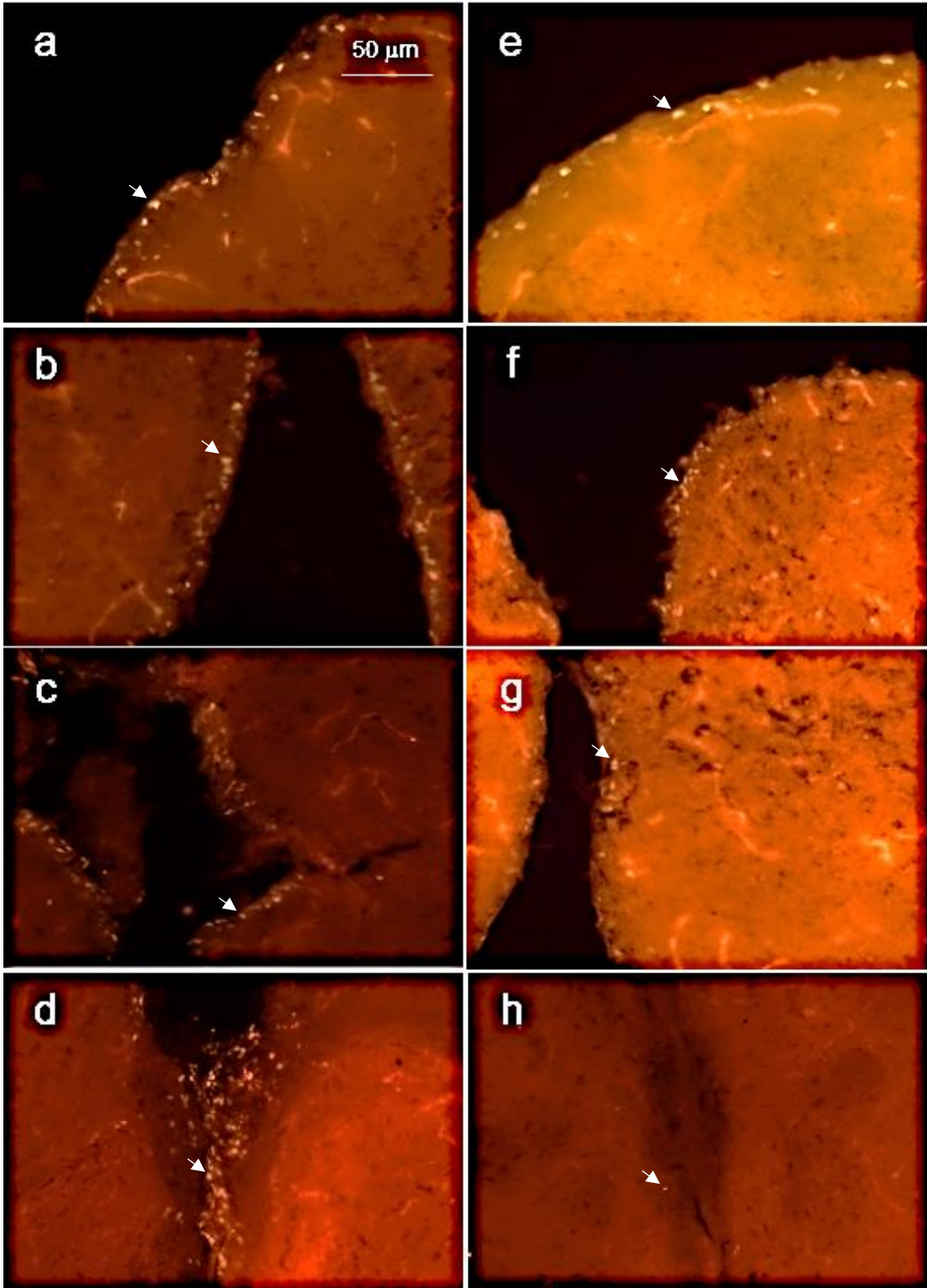


Figure 6. Brain images of wild-caught killifish. HP forebrain: (a) DL, (b) DM, (c) V, and HP midbrain: (d) PVZ. RO forebrain: (e) DL, (f) DM, (g) V, and RO midbrain: (h) PVZ. White arrows directed at PCNA+ cells as a means of reference. Edits conducted in Word 2016 v. 16: sharpen (50%), brightness (+40%), and contrast (-20%).

In wild-caught populations of killifish, the density of proliferating cells (PCNA⁺ cells/mm³) was about twice the rate in HP population in comparison to the RO/LP populations (Table 2 and Fig 5). This trend is observed in each of the three rivers studied. Furthermore, the rate of brain cell proliferation is relatively the same in the three HP populations and likewise in the RO/LP populations. Thus, within a stream, HP fish had significantly higher PCNA⁺ cell density than RO/LP fish in all brain regions.

Common Garden Study. The F1 reared fish for the common garden experiment showed similar population and region differences in cell proliferation as fish from the wild-caught populations. Fish from HP genetic lines had significantly greater PCNA⁺ cell density than fish from RO derived fish (d.f. = 1, F = 78.4, P = <0.001; Table 3). The various brain regions were also statistically different (d.f. = 3, F = 5.63, P = <0.005; Table 3). Similarly, the HP cross-sections (Fig 8a-d) show an abundance of PCNA⁺ cells and a lack of PCNA⁺ in the RO fish (Fig 8e-h). The consistency between the wild-caught populations and the lab reared fish shows that the natural population differences persist in a lab setting.

Table 3. ANOVA results showing the effect of predation environment on the density of proliferating cells in four brain regions of lab-reared fish.

effect	d.f.	<i>F</i>	<i>P</i>
brain region	3	5.63	<0.005
stream	1	0.03	>0.05
predation	1	78.4	<0.0001
brain region X stream	3	0.51	>0.05
brain region X predation	3	1.53	>0.05
stream X predation	1	0.27	>0.05
brain region X stream X predation	3	1.70	>0.05

Table 4: Mean density (\pm SE) of proliferating cells (PCNA+ cell/mm³) in four brain regions in lab-reared killifish derived from populations with differing predation pressure.

Stream	Predation	N	Forebrain			Midbrain
			DL	DM	V	PVZ
Arima	RO	9	9365 \pm 2874	12204 \pm 2556	15818 \pm 3999	10211 \pm 1638
	HP	8	31540 \pm 6251	29926 \pm 5283	30239 \pm 7411	21864 \pm 2488
Aripo	RO	5	13529 \pm 3294	15302 \pm 3280	13033 \pm 5801	8195 \pm 1015
	HP	9	26961 \pm 2558	25876 \pm 3044	38763 \pm 4951	17079 \pm 2979

RO = *Rivulus* only (no predators); HP = High predator density; LP = low predator density, DL= dorsolateral telencephalon, DM= dorsomedial telencephalon, V = ventral telencephalon, PVZ = periventricular zone

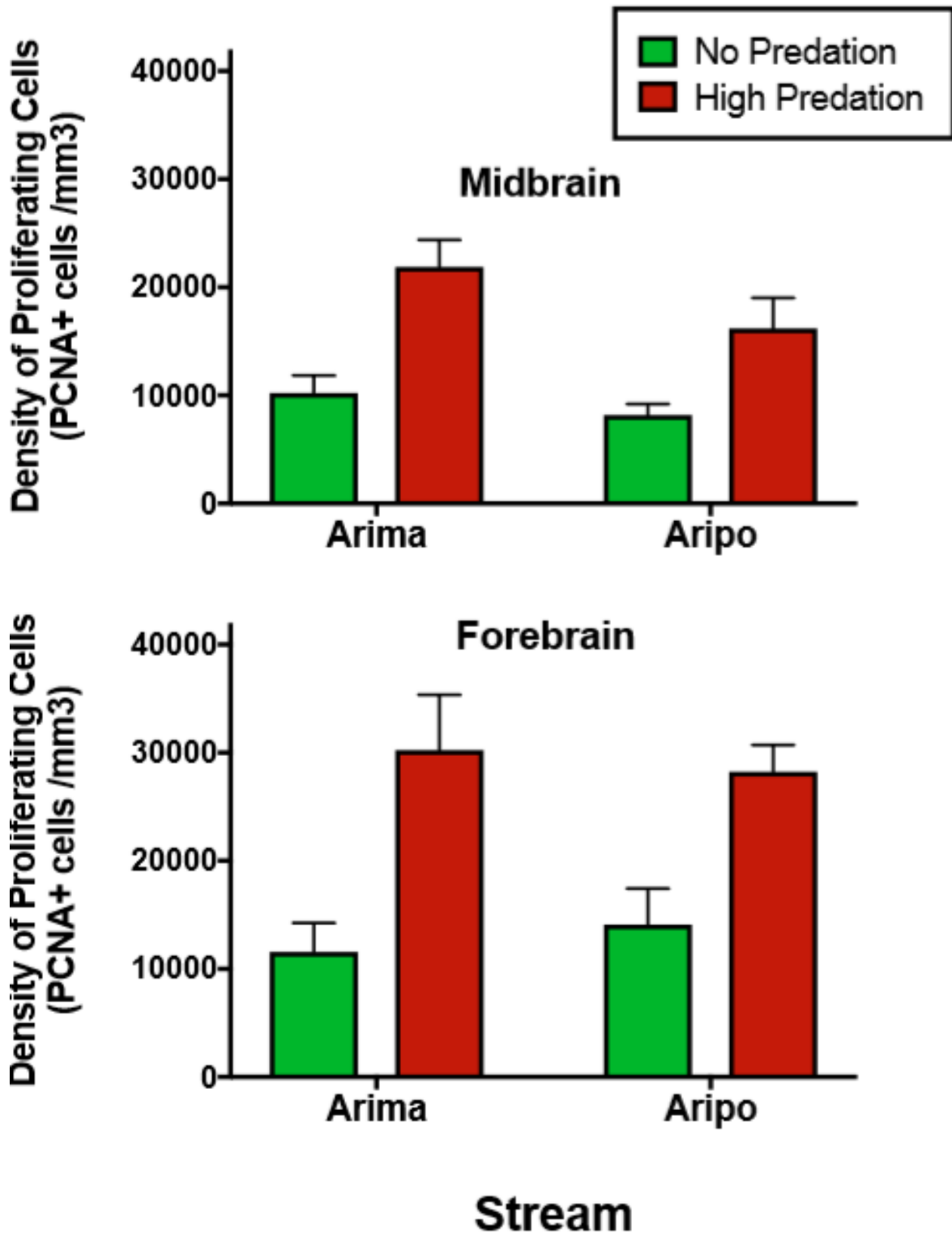


Figure 7. The density of cell proliferation in the forebrain and midbrain of lab reared F1 generation killifish derived from HP and RO populations in the Arima and Aripo River.

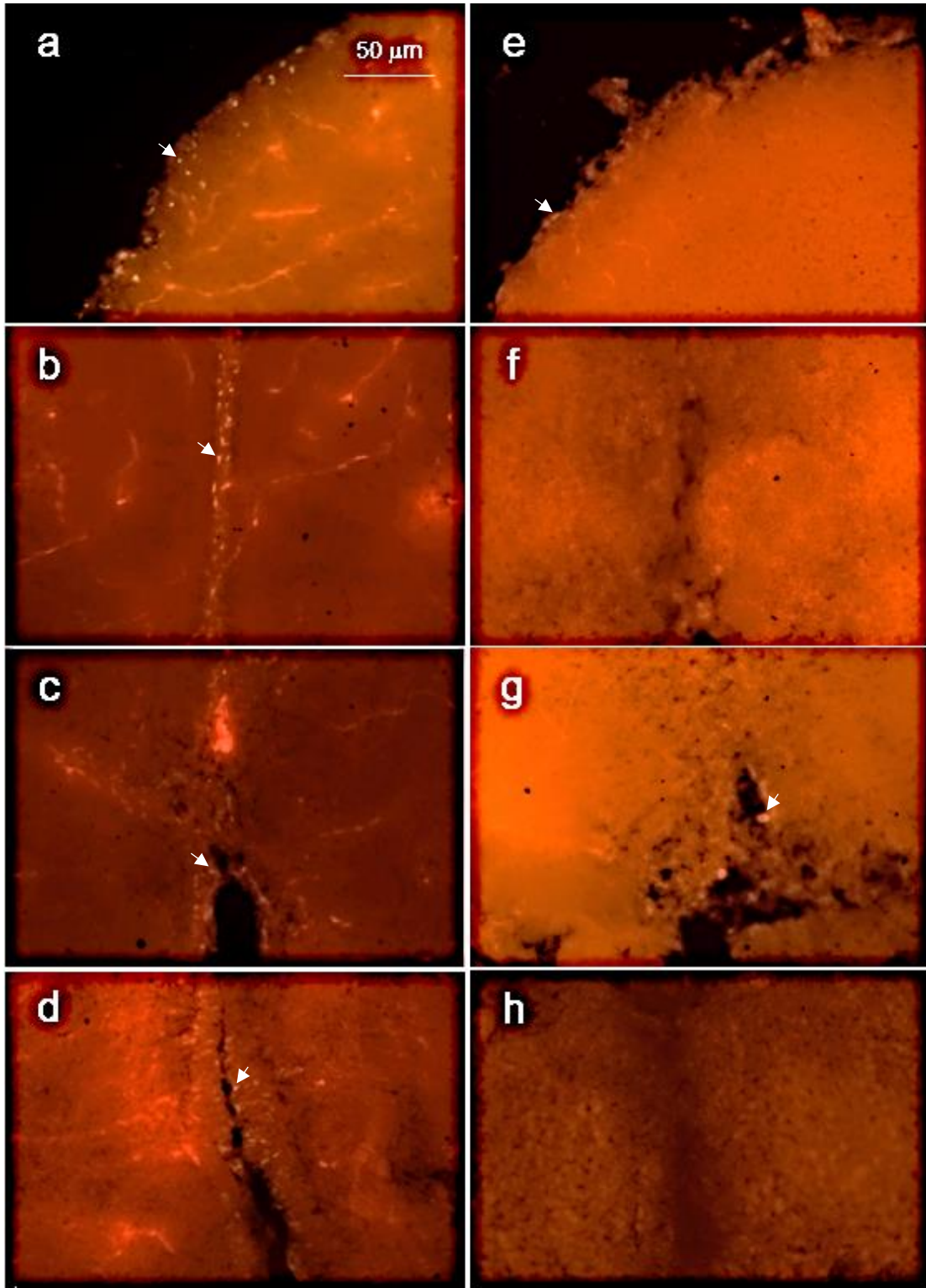


Figure 8. Brain images of captive killifish. HP forebrain: (a) DL, (b) DM, (c) V, and HP midbrain: (d) PVZ. RO forebrain: (e) DL, (f) DM, (g) V, and RO midbrain: (h) PVZ. White arrows directed at PCNA+ cells as a means of reference. Edits conducted in Word 2016 v. 16: sharpen (50%), brightness (+40%), and contrast (-20%).

(29) In the captive fish from the common garden experiment, the same trend in cell proliferation from wild-caught killifish is observed in the lab reared fish. Killifish derived from HP populations from the Arima and Aripo River have about twice the rate of cell proliferation as the fish derived from the RO populations (Table 4 and Fig 7). The rate of cell proliferation marginally decreased in the lab reared HP and RO derived fish in comparison to the wild-caught killifish from HP and RO/LP populations.

DISCUSSION

Here, I tested how predation affects brain cell proliferation in wild-caught and F1 lab reared Trinidadian killifish. Wild-caught killifish from HP populations had higher levels of brain cell proliferation than RO populations. Similarly, F1 fish from wild HP population reared in the common garden experiment presented with higher levels of brain cell proliferation than those from RO populations despite lack of any predator stimulus. Contrary to what has been seen in other species, the strong correlation between predator and brain cell proliferation suggests that the presence of predators select for brains with high levels of cell birth. Therefore, it appears that predation influenced Trinidadian killifish to evolve this trait through transgenerational selection – i.e., microevolution. In collaboration with previous work (Walsh *et al.*, 2016), our work suggests that high predation selects for smaller brains yet high levels of brain cell proliferation in Trinidadian killifish.

Phenotypic plasticity vs. intrinsic population differences

Phenotypic plasticity in response to predation is documented in an earlier set of studies on electric fish (Dunlap *et al.*, 2016) (Dunlap *et al.*, 2017). In field studies, *B. occidentalis* from Panama had decreased rates of brain cell proliferation when exposed to higher predator pressures (Dunlap *et al.*, 2016). Moreover, *B. gauderio* and *A. leptorhynchus*, had decreased rates of brain cell proliferation when exposed to lab stimulated predatory stimuli in the lab (Dunlap *et al.*, 2017). (A genetic component could not be assessed in the weakly electric fish study because these fish could not be bred in lab conditions.) However, this study showed the opposite pattern: fish living in high predation environments had elevated rates of brain cell proliferation.

The field study revealed that killifish from HP populations had increased brain cell proliferation; however, from the field study alone, I could not determine whether this response was a form of phenotypic plasticity, or if there was a genetic component to the differences in brain size and cell activity. The common garden experiment suggested that the differences seen were genetic rather than phenotypic because trait transmission occurred from parental generation to the offspring. That is, the F1 reared killifish had brain cell proliferation results that mirrored those from the field study despite not having a predator stimulus. That is, F1 fish derived from HP populations had twice the rate of brain cell proliferation as RO derived fish. This transgenerational expression of brain cell proliferation difference suggests a there is a genetic factor controlling the rate of cell birth.

Transgenerational inheritance can occur through standard genetic (Mendelian) mechanisms or through epigenetic inheritance. Epigenetic inheritance has been documented extensively in mammalian models (Rakyan *et al.*, 2003; Daxinger and Whitelaw, 2012; Heard and Martienssen, 2014). Epigenetic information can be inherited through mammalian germline, and it represents a plausible transgenerational carrier of environmental information (Carone *et al.*, 2010). Interestingly, this can be more specifically classified as paternal or maternal effect. Studies testing paternal epigenetics (Carone *et al.*, 2010; Wei *et al.*, 2014), observed how male mice influenced the genetic composition of their offspring. Dias and Ressler (2014) subjected F0 male mice to a fear odor right before conception and found that F1 and F2 male mice had increased sensitivity to that same fear odor. Moreover, another mouse study on transgenerational epigenetic programming concluded that sperm microRNA may be a bio-mechanistic explanation for how paternal traits manifest in the offspring (Rodgers *et al.*, 2015). Since the killifish used in this study were male, these mammalian studies support my conclusion – predator presence can genetically influence the rate of brain cell proliferation – because they document how alterations in the F0 male’s life history will influence the genomes of its offspring. That being said, the scope of my study cannot fully support epigenetic inheritance; therefore, population changes were caused by a genetic divergence due to natural selection.

Maternal factors are non-genetic effects provided by the mother can influence the development of an embryo. Since the female supplies the egg, in both viviparous and oviparous organisms, maternal factors can exist as proteins inside the egg that influence the genome of the embryo (Wolff *et al.*, 1998). Alternatively, traditional genetic

inheritance is prompted by selection pressure or mutations in the DNA that change population traits. Although such epigenetic mechanisms could potentially affect brain cell proliferation (Fagiolini *et al.*, 2009; Wilkinson *et al.*, 2007), my study cannot distinguish between epigenetics and traditional genetic inheritance. In my study, the killifish were of the F1 rather than the desired F2 generation. The F0 females were wild-caught prior to being acclimated into the common garden. This means that these F0 females were exposed to predators and other environmental stimuli during their life history. Thereby, the maternal factors may have influenced the development of the F1 killifish rather than the genes. Consequently, the epigenetic influence cannot be fully rejected from my results.

Brain Size Paradox

As stated previously, killifish from HP populations had smaller brains and increased brain cell proliferation, whereas, killifish from RO populations had larger brains and decreased rates of cell birth. These findings – i.e., smaller brains have twice as much cell birth than killifish with bigger brains – are paradoxical because if more cells are being added to the brain, one would expect a larger brain. (If more matter is added, there will be a total increase in matter.) There are two possible explanations that may resolve this brain size paradox: differences in brain allometry and increased rates of apoptosis in the brain.

It is possible that HP and RO brains are significantly different in size during earlier juvenile/adult years. The brain from HP fish may be significantly smaller due to slower brain growth before adulthood because, under high predation pressure, HP fish divert

energy to sexual maturation and growth (Walsh and Reznick, 2008). Selection by predators could genetically inhibit the rate of brain growth, similar to how it has increased the rate of brain cell proliferation. Therefore, HP killifish populations may have had lower rates of brain growth in the juvenile period, but increased brain growth relative to body size during early years of life to “catch up” to the brains of RO killifish. This suggestion is supported by data on brain allometry conducted in parallel to this thesis: Dunlap, Corbo, Vergara, Beston, and Walsh (submitted)

Another possible explanation is that the rate of cell death far surpasses the rate of cell proliferation in the brain. Since killifish from HP populations live in a stressful environment, cell death may be increased by high levels of cortisol or other stress-related signals which may then increase apoptosis (Jacobs *et al.*, 2000). In response to increased apoptosis, the rate of cell proliferation might significantly increase to replace apoptotic cells. Neural plasticity is generally managed by balancing the rates of cell proliferation and cell death (Krumschnabel and Podrabsky, 2008). Previously, it was thought that apoptosis occurred predominantly during embryogenesis, but later work reveals that apoptosis continued into adult teleost fish (Soutsckek and Zupanc, 1995; Soutsckek and Zupanc, 1996; Zupanc *et al.*, 1998; Zupanc, 2008). This understanding of neural plasticity suggests that there is a high level of cell proliferation to compensate for the high rate of apoptosis in the brain of killifish from HP populations.

Future Directions

Few studies have examined brain cell proliferation rates in natural populations, which makes this present study important for the reasons discussed. Moreover, I have explored

how cell proliferation rate is transgenerational. To better understand how proliferation rate is inherited, more epigenetic studies are needed. Such studies would further our understanding of neural plasticity, epigenetics, and phenotypic plasticity to environmental stimuli. Traditional genetic inheritance seems the sufficient explanation for my study, but I cannot rule out phenotypic plasticity. Testing for a phenotypic response requires exposing the wild-caught killifish to lab predator stimuli, similar to the predator cues used previously (Dunlap *et al.*, 2017). If the RO lab reared fish had increased brain cell proliferation after given a lab simulated predator experience, that would complicate the genetic explanation purported. In addition, my conclusions could be tested more fully by examining successive generations to rule out possible maternal effects transmitted into the F1 generations.

Cell death studies ought to be considered, especially for understanding neural plasticity in the Trinidadian killifish. Several caspase markers were tested on these brain tissues; however, the results were inconclusive due to a lack of confidence in the amount of positive caspase labeling occurring. Labeling DNA fragmentation *via* TUNEL assays has been a successful cell death procedure used in other fish studies (Soutsckek and Zupanc, 1995; Soutsckek and Zupanc, 1996; Zupanc *et al.*, 1998). TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling – measure the amount of DNA fragmentation in cells as DNA breaks during apoptosis (Kraupp *et al.*, 1995). A successful cell death study would clarify and answer my questions about the relationship between cell death and cell birth in the brains of killifish. For now, to explain the differences in brain size between HP and RO killifish, I postulate that predators cause an increased rate in brain cell proliferation and a much greater rate of cell death in brains of Trinidadian

killifish. This increased rate of cell death could explain why the HP brains are smaller than the RO as discussed in concurrent study (Dunlap, Corbo, Vergara, Beston, and Walsh; submitted)

Theoretically, this type of study can be used as a model to understand how families are impacted by living in stressful conditions. For example, consecutive generations living in low poverty (or any stressful environment) may be genetically expressing different genes in comparison to a family that has grown up well-off and healthy. Conducting epigenetic studies on neurogenesis may could potentially explain how biological changes create boundaries in society in demographics and socioeconomic status.

CONCLUSION

Exposure to high level of predator selection was to shown to reduce the size of the brains of Trinidadian killifish (Walsh *et al.*, 2016), as well as significantly increase the rate of brain cell proliferation. Because these results were present in the wild-caught and common garden experiment, it suggests that predators genetically select for these phenotypes. Thus, this transgenerational effect documents microevolutionary changes in response to predation.

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