HOMEOSTATIC REGULATION OF ACCESS SEROTONIN IS SUGGESTED BY AN INCREASED NUMBER OF SEROTONIN TRANSPORTER (SLC6A4A) EXPRESSING NEURONS OF THE DORSAL RAPHE

A thesis submitted in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

to the faculty of the

DEPARTMENT OF BIOLOGICAL SCIENCES

of

ST. JOHN'S COLLEGE OF LIBERAL ARTS AND SCIENCES

at

ST. JOHN'S UNIVERSITY

New York

by

Hala AlGarni

Date submitted: 3/31/ 2025 Date Approved: 4/2/2025

Hala AlGarni

Robert Kozol, PhD

ABSTRACT

HOMEOSTATIC REGULATION OF ACCESS SEROTONIN IS SUGGESTED BY AN INCREASED NUMBER OF SEROTONIN TRANSPORTER (SLC6A4A) EXPRESSING NEURONS OF THE DORSAL RAPHE

Hala AlGarni

Serotonin is a neurotransmitter system that is essential for driving behavior related to movement, attention and foraging. In humans, an increase or decrease in healthy levels of serotonin result in neuropsychological issues, including anxiety and depression. Despite these health concerns, our understanding of serotonin regulation and homeostasis is still poorly understood and needs more research from a variety of model and non-model animal systems. This study was designed to study serotonin homeostasis in the blind Mexican cavefish, a non-model system for understanding complex biological traits. The blind Mexican cavefish provides a multi-population species that includes river dwelling fish with eyes, that are considered ancestral, and cave derived populations that lack eyes and pigment. Previous research found that cavefish exhibit increased levels of serotonin that is hypothesized to drive decreased sleep and increased activity in cavefish. We wanted to test the hypothesis that increased levels of serotonin are sequestered or degraded in the brain via genetic compensation. To test this hypothesis, we compared mRNA gene expression of the serotonin promoting enzymes *tryptophan hydroxylase 1* and 2 (*tph1* and *tph2*) between surface and cave larvae. However, we did not find significant differences in gene expression between the two populations. To further explore serotonin regulation, we quantified the number of cells in the locus coeruleus

expressing the serotonin transporter *solute carrier family 6 member 4* (*slc6a4a*). We did find a significant increase in neurons expressing *slc6a4a* in the dorsal raphe of cavefish. This research provides insight into the regulation of serotonin in a non-model species, the blind Mexican cavefish. Future work will functionally test whether free serotonin is sequestered more rapidly before being implemented in circuits.

DEDICATION

This thesis is dedicated to my beloved mother, Dr. Samira Adham, whose love and unwavering support shaped my life in ways I can never fully repay. Even after your passing nine years ago, you still remain the most present to me. Your presence continues to guide me until this day, and your strength remains my greatest source of inspiration. I am eternally grateful for the life I had with you, the lessons I learned from you, and the resilience I gained from this journey to continue pursuing a path we started together. This is as much your achievement as it is mine, and I hope I made you proud.

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to my committee for their time, continuous guidance, feedback, and support throughout this journey. I want to thank my mentor, Dr. Robert Kozol for his continuous guidance through this research. None of this would be possible without your unwavering support. Thank you for giving me the opportunity to work in your lab. I am grateful for all my lab colleagues who took the time to support me through my journey. I would like to thank my father Abdullah AlGarni, who despite the distance, went above and beyond to support me in every way possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Serotonin, (5-hydroxytryptamine), is a major neurotransmitter involved in regulating cognitive functions including mood, cognition, memory, and sleep [1-3]. Serotonin is highly conserved in taxa, from invertebrates to chordates, as a neurotransmitter and signaling pathway for basic functions [2]. The serotonergic system evolved to control basic movements and feeding behaviors [1-4], that are essential in organisms like nematodes [4], while expanding in vertebrates to specialize in sensory processing [2], learning and memory [2, 5]. While this diversity of function is well studied, it remains unknown how drastic changes in an organism's environment shape the serotonergic system and its relation to essential behaviors.

Cave habitats provide extreme environmental variables, from no light to anoxia, that limit species recruitment and species diversity [6]. Organisms that are adapted to surface conditions must adapt via selective pressures for life in a extreme environment, that is best characterized by troglobitic traits, like eye, pigment and sleep loss [7]. For nearly a century, researchers have used the blind Mexican Tetra, *Astyanax mexicanus*, as a key model for investigating how standing genetic variation impacts biological traits through the process of evolution [7-9]. This species is characterized by two distinct morphs, a river dwelling surface fish that maintains ancestral features and cave adapted populations that have convergent troglobitic features, such as eye degeneration and sleep loss [10, 11]. While these attributes provide an ideal organism to model evolution in vertebrates, the cavefish field has also developed model organism tools, such as sequenced genomes [12], transcriptomic atlases [8], CRISPR-Cas9 [13], transgenic lines for live brain imaging [14] and a host of computational tools [15, 16]. However, the most important attribute is

population hybridization, that allows researchers to identify allelic variation that are important genetic discoveries for basic biology and biomedical research [7].

One early genetic discovery in cavefish was coding mutations in the enzyme Monoamine Oxidase (MAO) of several independently evolved cavefish populations [17, 18]. The prominent P106L mutation in cavefish monoamine oxidase (MAO) resulted in a partial loss-of-function, leading to significant alterations in brain monoamine homeostasis [19]. This mutation results in elevated serotonin levels of the brain, that has been hypothesized as a major driver in the evolution of cavefish behaviors [19, 20]. While these studies suggest the serotonergic system has been rewired in cavefish, we still do not know how serotonin homeostasis is maintained in cavefish and whether compensatory mechanisms exist to buffer access serotonin in the brain of cavefish.

This master's thesis was designed to determine whether larval cavefish exhibit genetic compensation to regulate increased levels of serotonin in the brain. To investigate how serotonin is regulated in cavefish, we utilized two complementary genetic assays, quantitative PCR and *in situ* hybridization of serotonergic genes in surface fish and cavefish larvae. We found that while cavefish do not exhibit a significant change in gene expression of serotonin production enzymes, they do exhibit an increase in the amount of serotonin transporter expressing cells of the dorsal raphe. This research provides valuable insight into the genetic mechanisms driving serotonin evolution in cavefish and future research will utilize functional testing of serotonergic genes and the dorsal raphes to test how these changes impact adaptive behaviors in cavefish.

METHODS

Animal Care and Husbandry

Adult populations of *Astyanax mexicanus* were maintained and cared for according to the IACUC approved protocol 2073. Fish are maintained on a recirculating rack in the St. John's University cavefish facility located in the sub-basement of St. Albert Hall Room G008. Fish are kept at 23 °C on a 14:10 hour light and dark light cycle. Larvae used in this study were generously proved by Dr. Johanna Kowalko from Lehigh University.

RNA Extraction

Whole RNA was extracted from groups of surface and cavefish larvae using the TRIzol Reagent RNA extraction protocol. In brief, 20 larvae were anesthetized in 50 mg/50 mL of MS222 and then homogenized using a 1.5 mL microcentrifuge tube mortar and pestle in 50 µL of TRIzol reagent. Homogenized samples were then brought to 1 mL of TRIzol reagent, mixed thoroughly and frozen at -20 C. Following 24 hours of freezing, 180 µL of chloroform were added to the samples, centrifuged at >12,000 RPM for phase separation and aqueous phases were collected in a new tube. RNA was then precipitated using ethanol extraction, quantified on a nanodrop 1000 and qualitatively assessed on an electrophoretic gel.

cDNA Synthesis

1 microgram of whole RNA was added to the iScript cDNA synthesis kit. Synthesis reactions were run at 25 °C for 5 min, 48 °C for 20 min and 95 °C for 1 min. Turbo DNAse was then added to the reactions and heated at 37 °C for 15 min. cDNA samples were then nano dropped to determine concentration and then diluted to 20 ng/µL for RT-qPCR experiments.

Quantitative PCR

qPCR was run using the PowerUp® Clear MasterMix (Thermo Fisher, MA, USA) on a Bio- Rad CFX 96 Real-time System. qPCR reactions were prepared as follows; 5 µL 2X SYBR green, 0.5 µL 20 nM forward/reverse primer, 1 µL 20 ng/µL cDNA template and 3.5 µL of RNAse/DNAse free water. Reactions were run in triplicate and plates contained the following samples: five dilutions of purified PCR product as a standard curve, no template control, no reverse transcription control and three independent unknown samples for surface and cave populations. Three independent samples were run for both Rio Choy surface fish and Pachón cavefish. Samples were run through the following thermal conditions: 95 °C for 3 minute, 40 cycles; 95 °C for 15 seconds, 58 °C for 20 seconds; 65-95 °C thermal cline (0.5 °C/s) to record melting curves. Fluorescence intensity was collected and recorded following each PCR cycle. Plates and samples were evaluated for accuracy via sample variance (replicate variance < 1 Ct), PCR efficiencies (e=80-110%) and NTC/NRT controls (Ct>39). Ct values were then imported into custom excel sheets to calculate delta-delta Ct and fold changes.

Delta-Delta Ct Analysis

Ct values for each sample were collected before calculating the change in Ct between the target gene and the reference gene for each sample. The following equation was used to calculate delta Ct

$$\Delta CT = CT\ (Target\ Gene) - CT\ (Reference\ Gene)$$

These delta Ct values were then used to calculate the delta-delta CT values which compute the difference in delta CT between the target gene and reference gene.

$$\Delta\Delta CT = \Delta CT\ (a\ target\ sample) - \Delta CT\ (a\ reference\ sample)$$

$$= (CTD - CTB) - (CTC - CTA)$$

Statistical significance was then calculated as a student's two-tailed t test between control sample and unknown sample delta-delta CT values for each target gene. Delta- delta Ct values were then converted into a fold change of the target gene expression to a reference sample via reference gene normalization.

In Situ Hybridization

Surface fish and cavefish were grown to 6 days post-fertilization and then euthanized in 50 mg/50 mL of system water for 15 minutes. Larvae were then fixed overnight in 4% formaldehyde/1X PBS at 4 °C. The following day, larvae were washed and dehydrated in multiple washes of 100% MeOH. Following dehydration, larvae were incubated in prehybridization buffer and then hybridized in RNA probe overnight at 37 °C with shaking. The probe was then washed-out using probe wash buffer and 5x SSCT, before acclimating in an amplification buffer. Finally, larvae were amplified overnight using 555 nm and 643 nm hairpin mixtures. All probes and hairpins were designed and manufactured by molecular instruments.

Confocal Imaging

In situ hybridized larvae were imaged using a Nikon A1R confocal microscope with a 20X, 1.0 NA, water dipping objective at 4x zoom. Imaged stacks were then down sampled to 512x512 microns and analyzed using the cell counter insert in FiJi ImageJ. Final counts were exported as txt files and analyzed using PRISM (Graphpad, inc).

Statistical Analysis

RT-qPCR delta-delta Ct values and cell counts were compared across surface and cave populations using a standard t-test in PRISM (Graphpad, inc).

RESULTS

Enzymes in the serotonin synthesis pathway do not exhibit genetic compensation

Previous work demonstrated that mutations in the *mao* gene cause increased levels of serotonin in larval cavefish. To determine whether cavefish exhibit compensation at the level of serotonin synthesis enzymes, we performed qPCR on the gene's *tryptophan hydroxylase 1* and *tryptophan hydroxylase 2 (tph1 and tph2*). We found that cavefish exhibit no difference in gene expression of *tph1* compared to surface fish at 6 days post fertilization (t=2.700, df=4, p=0.0541, Figure 1&2, Table 1). Alternatively, we found a slight increase in gene expression of cavefish *tph2* compared to surface fish, although it did not reach the threshold of statistical significance, (t=2.519, df=4, p=0.0654, Figure 1&2, Table 2). These data show that cavefish do not show a significant change in serotonin synthesis enzyme expression at the level of mRNA.

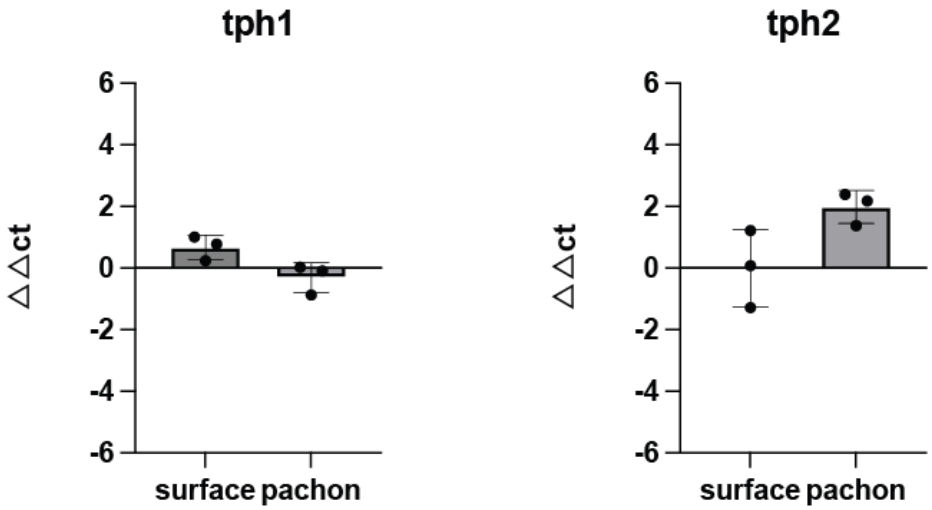

Figure 1. *tph1* and *tph2* qPCR gene expression comparison between surface and cavefish larvae. Bar graphs displaying the delta-delta Ct values of triplicate samples for the genes *tph1* and *tph2*. Both genes were not significant when comparing the delta-delta Ct values

between surface and cavefish samples. Samples were compared using a unpaired students t test.

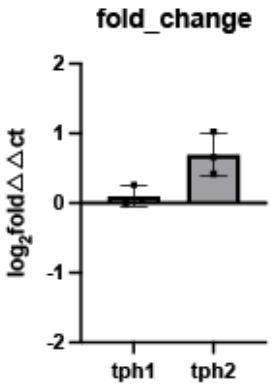

Figure 2. Cavefish exhibit slight increases in *tph1* and *tph2* gene expression. Bar graphs displaying the fold changes calculated from cavefish delta-delta Ct values for the genes *tph1* and *tph2*.

Table 1. Unpaired t-test comparing delta-delta Ct values for *tph1*

Upaired t test	
P value	0.0541
P value summary	Ns
Significantly different (P < 0.05)	No
One- two-tailed P value	Two-taied
t, df	T=2.700, df=4
Mean of column A	0.6676
Mean of column B	-0.3122
Difference between means (B - A) ± SEM	-0.9798 ± 0.3628
95% confidence interval	-1.987 to 0.02762
R squared (eta squared)	0.6458
F test to compare variances	
F, DFn, Dfd	1.524, 2, 2
P value	0.7923
P value summary	ns
Significantly different (P < 0.05)	No
Data Analyzed	
Sample size, column A	3
Sample size, column B	3

Table 2. Unpaired t-test comparing delta-delta Ct values for *tph2*

Upaired t test	
P value	0.0654
P value summary	Ns
Significantly different (P < 0.05)	No
One- two-tailed P value	Two-taied
t, df	T=2.519, df=4
Mean of column A	-1.00E-08
Mean of column B	1.981
Difference between means (B - A) ± SEM	1.981 ± 0.7864
95% confidence interval	-0.2027 to 4.164
R squared (eta squared)	0.6133
F test to compare variances	
F, DFn, Dfd	5.469, 2, 2
P value	0.3092
P value summary	ns
Significantly different (P < 0.05)	No
Data Analyzed	
Sample size, column A	3
Sample size, column B	3

Cavefish express more serotonin transporter (slc6a4a) positive cells in the dorsal raphe

While we did not observe variation at the genetic level of serotonin synthesis enzymes, we wondered whether components of serotonin recycling and degradation were upregulated in cavefish. To quantify variation in serotonin transport, we performed *in situ hybridization* for the serotonin transporter (slc6a4a) and quantified cell number in the dorsal raphe. We found that cavefish exhibit a significant increase in slc6a4a positive cells of the dorsal raphe ($t=4.996$, $df=7$, $p=0.016$, Figure 3, Table 3). These results show that cavefish exhibit more cells expressing serotonin transporter that may sequester larger amounts of serotonin.

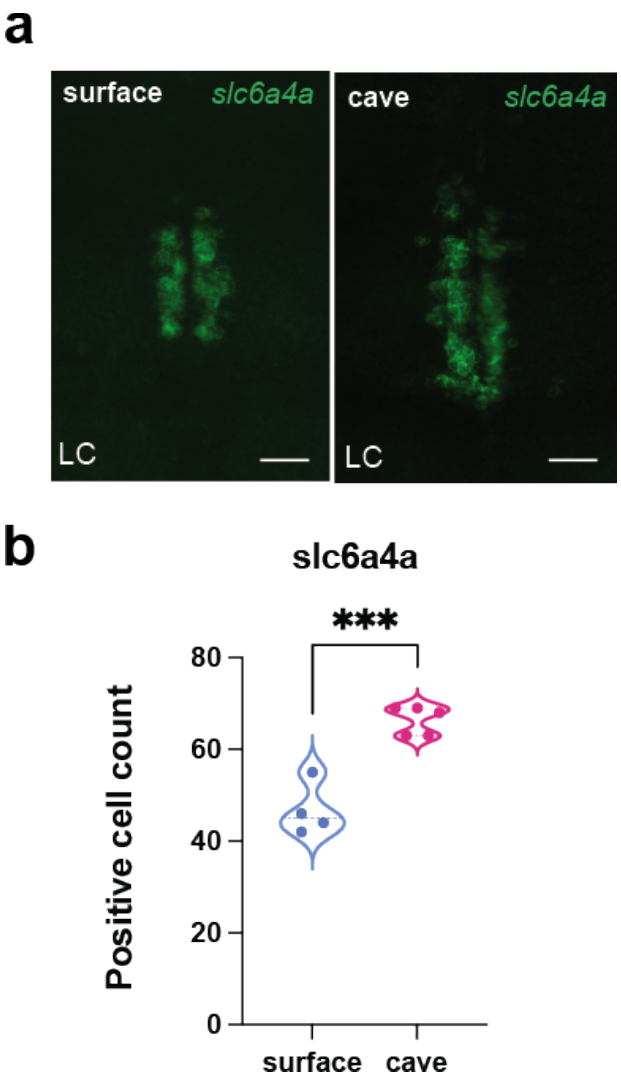

Figure 3. Cavefish exhibit an increase in the number of *slc6a4a* positive cells.

A. Confocal z-stack projections displaying the locus coeruleus in 20 micron flattened stacks. LC = locus coeruleus; Error bar = 20 microns. **B.** Violin plot displaying the cell counts for *slc6a4a* positive cells in the locus coeruleus. Cell counts were statistically compared using a unpaired students t test. $p>0.005$. Sample size; surface = 4, cavefish = 5.

Table 3. Unpaired t-test comparing cell counts of *slca4a6*

Upaired t test	
P value	0.0016
P value summary	**
Significantly different (P < 0.05)	Yes
One- two-tailed P value	Two-tailed
t, df	T=4.996, df=7
Mean of column A	47.50
Mean of column B	64.60
Difference between means (B - A) ± SEM	17.10 ± 3.422
95% confidence interval	9.007 to 25.19
R squared (eta squared)	0.7810
F test to compare variances	
F, DFn, Dfd	7.598, 3, 4
P value	0.0793
P value summary	ns
Significantly different (P < 0.05)	No
Data Analyzed	
Sample size, column A	4
Sample size, column B	5

DISCUSSION

Our understanding of serotonin homeostasis is paramount for determining how serotonin dysregulation impacts the behavior of animals and human health. While overabundance of serotonin in humans is known to adversely impact mental health, increased levels of serotonin have been theorized to be adaptive for cavefish, by contributing to a suite of behavioral features. This study sought test the hypothesis that increased serotonin causes genetic compensation at the level of serotonin producing enzymes and transporters, gene classes that have been shown to buffer mutational loads in other neurotransmitter systems. This study provides a proof-of-concept for using multiple genetic assays to determine how changes in one gene impact the regulation of other genes within a neurotransmitter's gene family.

Increased expression of transporters may reduce free serotonin for signaling within the dorsal raphe

While we did not see changes in the gene expression of serotonin promoting enzymes, we did detect changes in the number of cells expressing the serotonin transporter *slc6a4a*, that may suggest increased sequestering of serotonin in the dorsal raphe. The literature is replete with examples of genetic compensation, when a mutation at one end of a complexes pathway causes up or downregulation of other genes within a complex to rescue an otherwise deleterious biological result [21, 22]. For example, mutations in the glycine transporter gene *glyt2*, causes downregulation of specific glycinergic receptor subunits [23]. This decrease in the mRNA expression of receptor subunits creates a higher threshold for firing of glycinergic neurons, that in turn causes individuals carrying those mutations to not have paralysis [23]. In the case of cavefish, a large increase in available

serotonin could be causing an increase in the number of cells expressing the serotonin transporter *slc6a4a.* This increase would pump access serotonin out of the synaptic clefts of the cells in the dorsal raphe and therefore decrease overall serotonin signaling in the brain. Much more work is needed to verify this result, including quantification of *slc6a4a* mRNA via qPCR and CRISPR-Cas9 mutagenesis experiments to functionally test how *slc6a4a* impacts behavior in these two populations.

Increased levels of serotonin may provide an expansion in functional capacity that improves foraging or reduces energy expenditure

Serotonin is a neurotransmitter system that is necessary for essential behaviors, such as motivated behavior and attention. Previously research has elucidated the relationship between serotonin levels and common behaviors, such as foraging, which is used by most animals to find food sources [2, 4, 20, 24, 25]. While several neurotransmitter systems promote inactivity for energy conservation, serotonergic neurons promote active locomotion and oppose inactivity following the initiation of a search strategy or chemoattractant paradigm [25]. For most organisms, an overabundance of serotonin may overexert activity and cause stress or poor health, while for cavefish this increase in activity may be beneficial for finding scarce food sources or for social contact for mating, because cavefish exhibit no social behaviors such as schooling or shoaling [20, 24]. While our results showed no significant change in serotonin promoting enzymes, the enzyme *tph2* did show an increase in expression, suggesting further promotion of increased serotonin levels. It will be necessary to provide serotonin depletion and promotion assays in surface fish and cavefish to test the theory that increased serotonin drives behavioral changes that are adaptive in cavefish.

Functional assays to regulate serotonin would provide proof that the serotonergic system impacts adaptive behavior

Another study investigated the connection between serotonin depletion and changes in behavior in rats. Results of the effects of serotonin depletion on rats' social behavior investigated the consequences of long-term, brain-wide serotonin depletion on both social behavior and cognitive function in rats [26]. Using a genetic model that results in the reduced availability of serotonin throughout the brain, researchers investigated how this depletion affects the rats' ability to interact socially and perform cognitive tasks such as learning and memory. Results concluded that serotonin depletion led to significant alterations in social behavior, with affected rats showing reduced social interactions, less engagement with changes in social hierarchy dynamics. In contrast, while serotonin depletion also impaired certain aspects of cognitive performance, such as memory and learning tasks, these cognitive deficits were less significant compared to the social deficits. This impact suggests that serotonin plays a more prominent role in regulating social behavior than cognitive function. The results highlight the importance of serotonin in shaping social interactions and provide an insight into how serotonin dysregulation might contribute to social and cognitive deficits observed in various psychiatric and neurodevelopmental disorders, such as autism and depression.

CONCLUSION

Cavefish provide a unique system for studying serotonin regulation and dysregulation that will inform our understanding of the brain and neurological disorders. Future research will be necessary to identify all genetic changes in cavefish regulating the serotonin neurotransmitter system and functionally evaluate how the synthesis, storage and release of serotonin drives the adaptive behaviors that have evolved in cavefish.

REFERENCES

1. Haase, J., et al., *Regulation of the serotonin transporter by interacting proteins.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 722-8.
2. Gillette, R., *Evolution and function in serotonergic systems.* Integr Comp Biol, 2006. **46**(6): p. 838-46.
3. Azmitia, E.C., *Serotonin and brain: evolution, neuroplasticity, and homeostasis.* Int Rev Neurobiol, 2007. **77**: p. 31-56.
4. Iwanir, S., et al., *Serotonin promotes exploitation in complex environments by accelerating decision-making.* BMC Biol, 2016. **14**: p. 9.
5. Salvan, P., et al., *Serotonin regulation of behavior via large-scale neuromodulation of serotonin receptor networks.* Nat Neurosci, 2023. **26**(1): p. 53-63.
6. Bradic, M., et al., *Gene flow and population structure in the Mexican blind cavefish complex (Astyanax mexicanus)*, in *BMC Evolutionary Biology*. 2012. p. doi: 10.1186/1471-2148-12-9.
7. Jeffery, W.R., *Cavefish as a model system in evolutionary developmental biology*, in *Developmental Biology*. 2001. p. 1-12.
8. Warren, W.C., et al., *A chromosome-level genome of Astyanax mexicanus surface fish for comparing population-specific genetic differences contributing to trait evolution.* Nat Commun, 2021. **12**(1): p. 1447.
9. Gross, J.B., *The complex origin of Astyanax cavefish*, in *BMC Evolutionary Biology*. 2012.

10. Jeffery, W., et al., *Prox 1 in eye degeneration and sensory organ compensation during development and evolution of the cavefish Astyanax.* Dev Genes Evol, 2000. **210**(5): p. 223-30.

11. Duboué, E.R., A.C. Keene, and R.L. Borowsky, *Evolutionary convergence on sleep loss in cavefish populations*, in *Current Biology*. 2011. p. 671-676.

12. McGaugh, S.E., et al., *The cavefish genome reveals candidate genes for eye loss.* Nat Commun, 2014. **5**: p. 5307.

13. O'Gorman, M., et al., *Pleiotropic function of the oca2 gene underlies the evolution of sleep loss and albinism in cavefish.* Curr Biol, 2021. **31**(16): p. 3694-3701 e4.

14. Lloyd, E., et al., *Blind cavefish retain functional connectivity in the tectum despite loss of retinal input.* Curr Biol, 2022. **32**(17): p. 3720-3730 e3.

15. Kozol, R.A., et al., *A brain-wide analysis maps structural evolution to distinct anatomical module.* Elife, 2023. **12**.

16. Kozol, R.A., et al., *Evolution of a central dopamine circuit underlies adaptation of ligh-evoked sensorimotor response in blind cavefish, Astyanax mexicanus.* BioRxiv, 2024.

17. Pierre, C., et al., *The enzymatic and neurochemical outcomes of a mutation in Mexican cavefish MAO reveal teleost-specific aspects of brain monoamine homeostasis.* J Exp Biol, 2023. **226**(14).

18. Pierre, C., et al., *A mutation in monoamine oxidase (MAO) affects the evolution of stress behavior in the blind cavefish Astyanax mexicanus.* J Exp Biol, 2020. **223**(Pt 18).

19. Elipot, Y., et al., *A mutation in the enzyme monoamine oxidase explains part of the Astyanax cavefish behavioural syndrome.* Nat Commun, 2014. **5**: p. 3647.

20. Retaux, S. and Y. Elipot, *Feed or fight: A behavioral shift in blind cavefish.* Commun Integr Biol, 2013. **6**(2): p. e23166.

21. Kozol, R.A., et al., *Function Over Form: Modeling Groups of Inherited Neurological Conditions in Zebrafish.* Front Mol Neurosci, 2016. **9**: p. 55.

22. Buglo, E., et al., *Genetic compensation in a stable slc25a46 mutant zebrafish: A case for using F0 CRISPR mutagenesis to study phenotypes caused by inherited disease.* PLoS One, 2020. **15**(3): p. e0230566.

23. Ganser, L.R., et al., *Distinct phenotypes in zebrafish models of human startle disease.* Neurobiol Dis, 2013. **60**: p. 139-51.

24. Elipot, Y., et al., *Evolutionary shift from fighting to foraging in blind cavefish through changes in the serotonin network.* Curr Biol, 2013. **23**(1): p. 1-10.

25. Lottem, E., et al., *Activation of serotonin neurons promotes active persistence in a probabilistic foraging task.* Nat Commun, 2018. **9**(1): p. 1000.

26. Alonso, L., et al., *Constitutive depletion of brain serotonin differentially affects rats' social and cognitive abilities.* iScience, 2023. **26**(2): p. 105998.

Vita

Name	Hala AlGarni
Baccalaureate Degree Washington	Bachelor of Science George University, District of Columbia, Major: Neuroscience
Date Graduated	May, 2023