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Exploring the Developmental Basis of Monogamy: A Comparative Study of FGF Gene Expression in Convict Cichlids and Zebrafish

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EXPLORING THE DEVELOPMENTAL BASIS OF MONOGAMY: A COMPARATIVE STUDY OF FGF GENE EXPRESSION IN CONVICT CICHLIDS AND ZEBRAFISH

by

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ABSTRACT

The study of monogamy in convict cichlids and what sets them apart from other fish is crucial for understanding why their mating behaviors differ, as these behaviors impact their survival. Despite the shared DNA and brain structure among most fish, convict cichlids exhibit uniquely distinct social behaviors. To ascertain why this is the case, a series of experiments were conducted to identify and compare the FGF gene expression in convict cichlid developmental stages to that of the zebrafish. The FGF morphogens FGF8b and FGF receptor 2 were chosen based on literature review, and the resulting gene expression patterns varied across embryonic stages, indicating a possible decrease in FGFr2 and an increase in FGF8b in convict cichlids compared to zebrafish.

Keywords: *Amatitlania nigrofasciata*, Monogamy, Convict Cichlids, Fibroblast Growth Factor, Gene Expression, Oxytocin, Midbrain-Hindbrain Boundary, Hypothalamus

Dedication

To *Bangtan Sonyeondan* (BTS), for keeping me sane through the pandemic.

"The morning will come again,

No Darkness, No Season is Eternal." – *Spring Day*

ACKNOWLDGEMENTS

I would like to express my gratitude to Dr. Sylvester, who has been an outstanding teacher, mentor, and supervisor, providing sound guidance and support with the proper balance of insight and humor. A special thank you goes out to Dr. Margo Brinton and the graduate students in the Brinton Virology research lab. I gained a great deal of knowledge while working as a lab assistant, which aided me significantly throughout my independent research.

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I would like to convey my heartfelt appreciation to my sister, Ramsha, for always being there for me and reminding me that I am awesome even when I don't feel it. I appreciate you driving me to the lab at 3 a.m. simply so I could finish an experiment. Also, for letting me play BTS music in the car every morning. I'd like to express my sincere thanks to my brother, Areeb, for making me chuckle at the most inappropriate times.

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It is critical to maintain a healthy relationship with life beyond the abyssal depths of the lab. I cannot overstate the importance of the time I spent venting to my friends, Kareena and Alex. Your attentiveness means a lot to me.

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STATEMENT OF PROBLEM AND SIGNIFICANCE

Although monogamy is widely recognized at the molecular level in mature animals, its developmental foundation is still unknown. Throughout embryonic development, the expression of genes encoding external growth proteins and intracellular transcription factors regulates both brain compartmentalization and neuronal cell differentiation and migration (Kiecker & Lumsden, 2005). Variations in the intensity and timing of protein signaling generate distinct degrees of cell proliferation, growth, and apoptosis, leading to morphological and behavioral abnormalities in adulthood (Moreno & Gonzalez, 2011). Previously published work established that such heterogeneity in gene expression results in difference in the telencephalon patterning of two closely related convict fishes from Lake Malawi with divergent habitat preferences and that manipulation of the growth factors Hedgehog and Wingless is sufficient to replicate the forebrain structure of one or the other (Sylvester et. al., 2013). As a result, minor differences in patterning are sufficient to produce unique behavioral features. The aim of this research is to unravel the gene expression properties that contribute to the adoption of monogamous social systems in animals. To achieve this, Central American convict cichlids and South Asian zebrafish are utilized as comparative models for studying embryonic brain development. The experimental approach involves visualizing neuroendocrine hypothalamic markers using whole-mount in situ hybridization. The focus is on understanding how specific Fibroblast growth factors (FGF8b and FGFr2) lead to developmental differences in neuroendocrine hypothalamus neurons and hormone production between monogamous and non-monogamous species.

BACKGROUND

Survival of the fittest is the concept that describes the process of natural selection and, ultimately, reproductive success. It has been scientifically known and understood that various environmental factors, adaptations, and outside influences could impact how the offspring of organisms survive. One specific factor that can lead to an offspring's enhanced chance of survival is parental care, which is often found in monogamous relationships (Snekser & Itzkowitz, 2020). Monogamy is a type of behavior exhibited when an organism pairs with a mate for a long time and reproduces with that set mate for a long duration. This social species is established when a male adult and female adult pair cohabit within a shared nest and home range. The partners primarily engage in copulation with each other, while the males also participate in parental care. The male individuals additionally assume a guarding role to protect the nest from external threats. The pair bonds that are formed in monogamous species are durable. Studies have shown that monogamy can be caused due to low populations of organisms or increased reproductive efficiency; however, there are also known benefits of monogamy that do not involve reproduction or environmental factors and occur for other biological reasons (Whiteman & Cote, 2004).

When understanding the benefits of monogamy, one must understand how these organisms became monogamous. Various types of social organization include polygamy, which is the act of cohabiting with multiple partners, and promiscuity, which refers to the absence of long-term social relationships. Multiple experimental studies have been designed to analyze the intricacies of monogamy, including aspects such as partner selection, mate protection, and paternal care. The behavioral facets of monogamy can be elucidated by neuroscience. Various physiological and anatomical techniques can be utilized to examine the neural hormones that

govern monogamy. The hypothalamus is a brain region that contains a diverse population of neurons, each of which performs a unique function and has a distinct effect. The hypothalamus is responsible for a broader range of functions. It functions as the neuroendocrine system's central regulator, controlling critical homeostatic physiological processes such as thermoregulation, food intake, and circadian rhythms. It is also required to process a wide range of emotional and social actions, such as mating, fighting, and parental care (Blackshaw et al., 2010).

The hypothalamus has typically been labeled as the "feeding center" and the "pleasure center" due to the different types of neurons that it contains and the impact they have on maintaining homeostasis.

Fibroblast growth factor signaling in the developing hypothalamus

Fibroblast growth factor (FGF) signaling is essential for establishing several central areas. It controls the development of the neuroendocrine hypothalamus, which is a cluster of neuroendocrine neurons that originates largely in the nasal and ventricular zone of the diencephalon. The fibroblast growth factors (FGFs) are one of the most important signaling molecules involved in the orchestration of vertebrate brain development on a temporal and spatial scale. The FGF family comprises 22 highly similar peptides either secreted via a canonical or non-canonical pathway or operate intracranially within the nucleus or cytoplasm. Multiple studies have proven that they have a role in various central nervous system (CNS) processes, including neural induction, patterning, cell fate specification, neuronal survival, axon outgrowth, and cell migration (Tsai & Brooks, 2011).

The biological effects of released FGFs are predominantly mediated by four tyrosine kinase FGF receptors (FGFR1–4), which trigger various signal transduction cascades. The

significant deterioration of brain regions in transgenic mice that cannot produce FGFs, and their receptors serves as more evidence of the importance of secreted FGFs and their receptors in CNS development (Itoh & Ornitz, 2008).

FGF signaling pathway is known to be involved in developing several brain structures, including the telencephalon, diencephalon, midbrain-hindbrain junction, and the cerebellum (Mason, 2007). There are three zones in the embryonic head that are highly important for the development of a functional neuroendocrine: the olfactory placode, the telencephalon, and the ventral diencephalon. The neuroendocrine hypothalamus, which includes a cluster of essential neurosecretory neurons, is located within the ventral diencephalon. The VD promotes the maturation of most hypothalamic neurons, including oxytocin. The neurosecretory neurons are simultaneously projected to the median eminence (ME), neurohypophysis, and, in some circumstances, adenohypophysis in order to release hormones. The neuroendocrine hypothalamus secretes neurohormones that regulate various physiological functions, including reproduction, growth, metabolism, eating, and stress. During development, disruption of the neuroendocrine hypothalamus would most likely impair the organism's physiology, homeostasis, and, secondarily, cognitive function and emotional state (Tsai, 2011). There is substantial evidence for FGF signaling in these three zones during developmental phases (Tsai, 2011).

There is substantial evidence for FGF signaling in these three zones during developmental phases. FGF8 mRNA transcripts are present in the developing telencephalon's midline commissural plate, telencephalic/diencephalic border, and ventral diencephalon (prospective hypothalamus) during early human development (Fig. 1A). FGFR1, the primary receptor mediating FGF8 signaling, is similarly expressed in Rathke's pouch (Fig. 1, B, and D) and a larger area within the ventral diencephalon (Fig. 1, C, and D) (McCabe et al., 2011).

Sequential or simultaneous expression of many FGF signaling components suggests a complicated and occasionally redundant regulatory structure (Tsai, 2011).

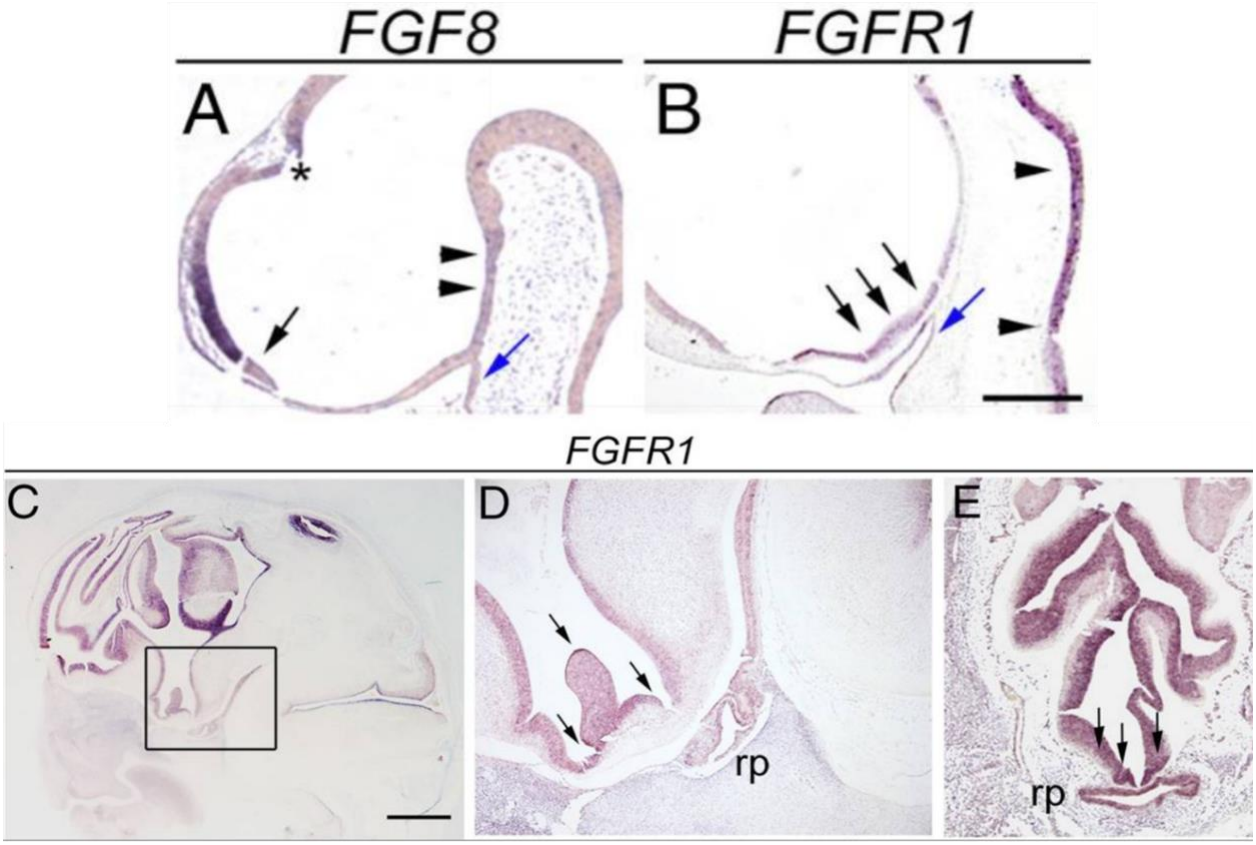

Figure 1. ***Expression of FGF8 and FGFR1 during early human development.*** *Expression of FGF8 (A) and FGFR1 (B–E) during human embryonic CS 16 [37 d after fertilization (A and B) and CS 22 (C, D, and E)]. Sagittal (A–D) and coronal sections (E) are shown. A, FGF8 transcripts are localized in the commissural plate of the telencephalon (arrow in A), telencephalic/diencephalic border (asterisk), and the* ***prospective hypothalamus (arrowheads in A)*** *but not in Rathke's pouch (rp, blue arrow), the primordium of the anterior pituitary gland. B–E, FGFR1 transcripts are widely detected in the* ***prospective hypothalamus (arrows in B, D, and E),*** *hindbrain (arrowheads in B), and the developing Rathke's pouch (blue arrow in B, rp in D and E). D (McCabe et al., 2011).*

Neuroendocrine Bases of Monogamy

Oxytocin is primarily produced in the hypothalamus. It is released into the bloodstream via the pituitary gland or to other areas of the brain and spinal cord, where it binds to specific receptors to alter behavior and physiology. Because of these characteristics, oxytocin has been grouped alongside other happy hormones – hormones believed to have a favorable effect on mood and emotions. The genes encoding oxytocin and its carrier protein, neurophysin I, are

expressed in the magnocellular neurons of the hypothalamic supraoptic (SON), paraventricular (PVN), and various accessory nuclei of the hypothalamus (Knobloch & Grinevich, 2014).

Investigations into the prairie vole population have revealed that the neuropeptide oxytocin plays a crucial role in mediating social behaviors. These behaviors encompass affiliation, parental care, and territorial aggression. Furthermore, oxytocin has been identified as a factor influencing the control of specific behaviors linked to monogamy (Young et al., 1993). The pharmacological data demonstrate that OT plays a role in monogamous behavior in prairie voles, while the same peptide has different effects in non-monogamous species (Oldfield & Hofmann, 2011).

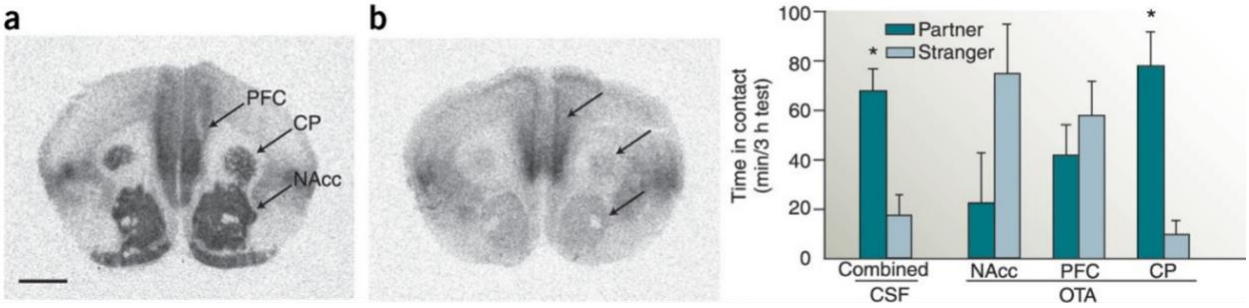

Figure 2. OTR regulation of pair bonding in prairie voles. *(a, b) Monogamous prairie voles (a) have higher densities of OTR in the nucleus accumbens (NAcc) and caudate putamen (CP) than do non monogamous montane voles (b). Both species have OTR in the prefrontal cortex (PFC). (graph) A selective OTR antagonist (OTA) infused bilaterally into the NAcc or PFC, but not the CP, blocks partner-preference formation in female prairie voles (Young & Wang, 2004).*

A comparison of the neuroanatomical distribution of OT receptors in monogamous prairie voles and anti-social montane voles reveals significant differences. As shown in Figure 2, Monogamous female prairie voles have a higher density of OT receptors in the nucleus accumbens and caudate-putamen, two nuclei of the basal ganglia, than non-monogamous montane voles. OT receptor is also present in the prefrontal cortex (PFC) of both species (Young & Wang, 2004). According to anatomical and pharmacological research, the prefrontal cortex and nucleus accumbens are key brain regions for pair-bond development. For instance, injecting an OT receptor antagonist into females' prefrontal brain and nucleus accumbens, but

not into their caudate-putamen, inhibits mating-induced partner preference formation. The ability to alter mate preference behavior shows that even minor changes to nonapeptide systems may substantially affect mating systems.

Sexual intimacy can increase oxytocin production, which helps adults' bond. In addition, the hormone isotocin, the fish homolog of oxytocin, has been found to briefly modify mating behavior in convict cichlids (Oldfield & Hoffman, 2011).

The Abnormal Hypothalamic oxytocin system in fibroblast growth factor-8 deficient mice

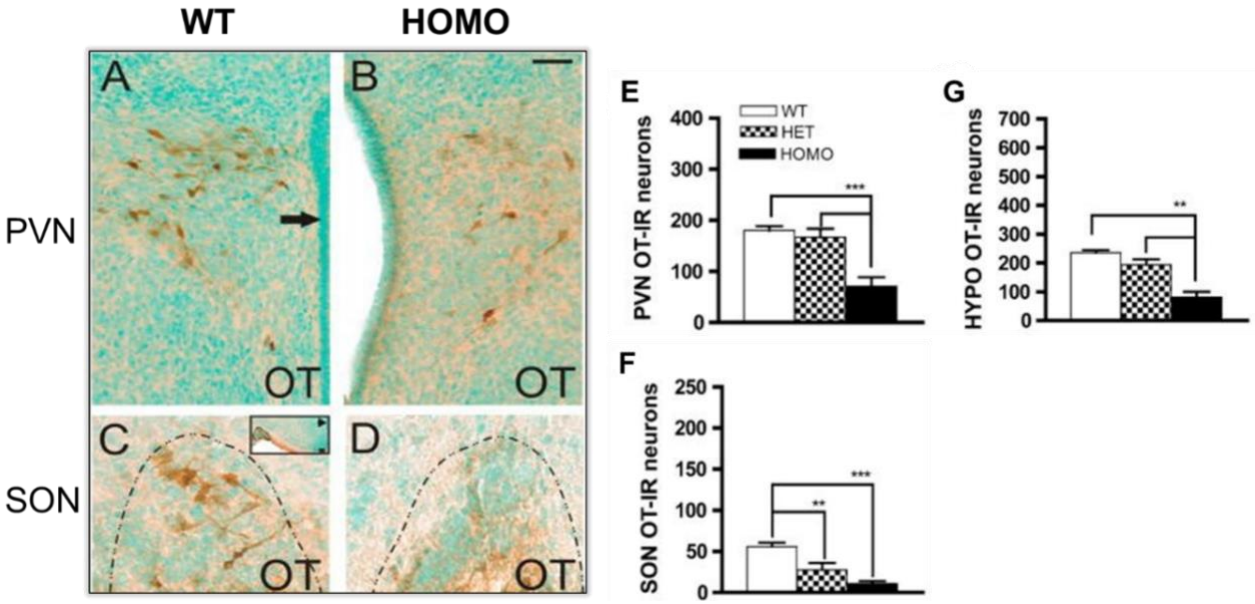

Figure 3. ***Representative photomicrographs of OT-IR neurons in the PVN (A, B) and SON (C, D) of WT (a, c) and HOMO (b, d) FGF8 hypomorphs.*** *Brown color represents OT immunostaining. Green color represents methyl green nuclear counterstain. Note the visual reduction of OT-IR neurons in (b) and (d) compared to (a) and (c). Quantification of OT-IR (G–I) neurons in WT and HOMO FGF8 hypomorphic mice. Data are represented as mean ± SEM. N = 6–7 per genotype. Significant difference between groups is indicated by brackets above bars. * P < 0.05, ** P < 0.01, *** P < 0.001*

The strongest indication that FGF signaling plays a role in the development of the mammalian neuroendocrine hypothalamus originates from a study on Fgf8 hypomorphic mice. The mice study investigated if fibroblast growth factor 8 (FGF8), a crucial signaling protein essential for forebrain growth, is essential for the correct development of the Oxytocin system. In contrast to their wild-type littermates, homozygous (HOMO) FGF8 hypomorphic mice exhibited

a considerable decrease in the number of neurons that were immunoreactive for the mature Oxytocin peptide in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Fig. 3)."

Overall, these findings imply that a lack of FGF8 results in a decrease in mature OT, which could be attributed to a decrease in prohormone synthesis or processing (Brooks, 2010). Moreover, as the presence of adequate hormone synthesis is a distinguishing feature of mature neuroendocrine neurons, this finding suggests that FGF8 signaling also plays a role in the phenotypic maturation of a neuroendocrine system arising from the diencephalon (Tsai & Brooks, 2011).

Convict Cichlid and Zebrafish as Comparative Model Organisms for the Study of the Developmental Basis of Monogamy

Although many animals form socially monogamous pair bonds, *Amatitlania nigrofasciata*, a biparental convict cichlid, provides a good non-avian paradigm for understanding monogamy. Research into the development of the brain and the formation of pair bonds is being conducted using this teleost fish. Although its genome is currently being sequenced, gene primers can be built using closely related species like *Oreochromis niloticus* (Nile Tilapia). Convict cichlids are serially monogamous, which means they look for new mates each mating season. This species has a distinctive social structure characterized by biparental care, alloparental care, and aggressive behavior against nest intruders (Snekser & Itzkowitz, 2019). *Danio rerio*, colloquially referred to as zebrafish, is a widely used model organism in animal research. Since the 1970s, it has been utilized extensively in research on vertebrate development. Its genome has been completely sequenced, and its small body size, big litter size, and quick developmental time (approximately three days) make it ideal for animal research.

General Rationale

This study aims to investigate the evolutionary divergences of fibroblast growth factor (FGF) genes involved in the brain development of monogamous convict cichlids. A series of tests were conducted to compare the gene expression of FGF ligands (FGF8b, FGF17, FGF2) and their receptors (FGFr1, FGFr2, FGFr3) between developmental stages of convict cichlids and zebrafish. The investigation begins by comparing gene expression in specific brain regions, utilizing isolation and visualization of FGF signaling molecules through whole-mount in-situ hybridization (WISH) in both monogamous convict cichlid fish and non-monogamous zebrafish. Identification of potential differences in gene expression enables quantification during specific embryonic stages, aiming to determine the factors underlying monogamy in convict cichlids. The study hypothesis proposes that the observed monogamous behavior in convict cichlid fish is attributable to elevated levels of FGF signaling morphogens, particularly in the hypothalamus, which in turn promote increased production of oxytocin hormones. This research aims to enhance understanding of the relationship between gene expression and the manifestation of monogamous behavior during the adult stages of convict cichlids.

METHOD

Experimental Plan: The purpose of these series of experiments is to create RNA probes to recognize gene expression in convict cichlids and zebrafish using in situ hybridization

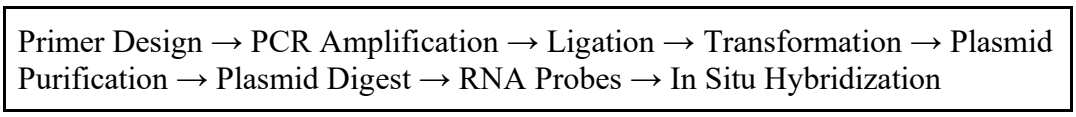

Figure 4. *A general template of the experimental plan*

A. Designing Primers

Primers are small pieces of RNA strands that act as binding sites for DNA polymerase during replication. Exon specific degenerate forward and reverse primers were designed for AC_βFGF, ON_FGF17, AC_FGFr1, AC_FGFr3, and DR_FGF8a using the NCBI gene database. Wet stock with concentration of 100 µM was created for each primer.

B. Polymerase-chain reaction and Gel Electrophoresis

PCR is a technique used to replicate gene sequences using complementary DNA (cDNA) and primers. cDNA sequences were made from zebrafish and convict cichlid embryos respectively. To maximize the chances that the primers will bind to the cDNA, nine different samples of cDNA for each convict cichlid gene and three samples for Danio rerio were used. PCR reaction was setup with total volume of 25.0 µL consisting of 12.5 µL GoTaq, 10.5 µL dH2O, 1.0 µL cDNA (match DNA and primer species), 0.5 µL primer_F, and 0.5 µL primer_R. PCR tubes were set in a thermocycler and the annealing temperature was modified to suit each primer. The size of the PCR products was verified using 1% agarose gel verification. After confirmation, PCR products were reamplified to produce sufficient amounts of amplicons.

C. Ligation and Transformation

Amplified gene sequences need to be inserted into a plasmid, a circular sequence of DNA, for future incorporation into bacterial cells. Ligation products are inserted into competent bacterial cells using the heat shock method. After growing the insert in AMP AGAR plates, the presence of insert was determined by the color of the colonies. The positive results were white, meaning successful insertion of insert into the plasmid, while the negative results were blue, or no presence of insert. It is preferable to make several ligation products with increased amounts of DNA insert while reducing dH2O. QIAGEN's QIAprep® Miniprep Kit was used to create minipreps for all the genes. Final concentrations between 20-100 ng/µL are ideal. Anything greater than 100 ng/L is splendid but must be confirmed with a PCR. The objective of making the miniprep is to isolate the recombinant plasmid DNA, containing the convict cichlid cDNA.

D. Plasmid Digest and RNA Probes

Using restriction enzymes NcoI and SacI, DNA was cleaved at the T7 and SP6 sites, respectively, to determine the anti-sense and sense probe. The SP6 and T7 RNA polymerase promoters can be used to synthesize RNA in vitro from purified plasmid DNA using either SP6 or T7 RNA polymerase. These promoters are antagonistic to one another and surround the cloning site. As a result, either strand of the cDNA clone can be transcribed into RNA. This step was used to make RNA probes, which attaches to the mRNA produced by transcription-activating genes. RNA is highly volatile, so strict technique was observed and the use of RNAse free water was crucial. The use of the riboprobes is to change the DNA to RNA, to bind the mRNA that is within the fish embryo. The RNA probe will be a sequence that complements the mRNA, and this can be seen throughout the process of whole-mount in-situ hybridization (WISH).

E. Whole-mount In situ Hybridization

In situ hybridization was used to stain the genes of interest to provide a visual aid. This technique involved using previously labeled complementary RNA to bind to a target RNA sequence within cells of interest. The embryos were rehydrated first in a series of graded washes: 1 x 5 minutes in 75% methanol/PBST, 50% methanol/PBST, 23 25% methanol/PBST and twice for 5 minutes in PBST. Proteinase K digestion was used at a 1:3000, 1:4000, and 1:5000 dilution and the cells were refixed by rinsing in PBST, then bathed in 4% PFA for 30 minutes. The embryos were washed 3 times with PBST and were now ready to bathe in a prehybridization buffer. After the incubation period, the prehybridization solution is replaced with a hybridization solution containing the probe and incubated overnight at 70ºC in a water bath. The next step is the antibody coupling step. The probe solution is removed and kept for future use. Kept at 70ºC in a water bath, the following washes were performed: washed 2 x 5 minutes in prehybridization solution, washed 1 x 5 minutes in 25% prehybridization/75% 2X SSC, washed 1 x 10 minutes in 2X SSC, washed 3 x 30 minutes in 0.2X SSC. At room temperature, the embryos were washed twice in MABT. A blocking solution washed the embryos for 2 hours on a rocker at room temperature. The blocking solution was replaced with a fresh blocking solution and the AP ANTI-DIG antibodies (1:3000 dilution) were added to the fresh blocking solution in each well. This was incubated overnight at 4ºC on a rocker.

After overnight incubation, a series of washes were performed beginning with a wash in MABT twice. After MABT, the embryos were washed every hour for six hours or more with TST at room temperature. The embryos are incubated overnight at 4ºC on a rocker. It is important to note that TST should remain at pH 9.0 to ensure the color reaction step proceeds as expected. The signal development step is the last step before microscopy. In this step, the

embryos were washed twice with NTMT. Again, NTMT should always be kept at pH 9.0. The substrates NBT and BCIP are added to the wells and placed in the dark to incubate at room temperature. The color should gradually change from translucent to purple on the areas of the embryos where the gene expression is expected. Once pink/purple coloration appeared the reaction was stopped by washing the embryos twice in PBS and the cells were refixed in 4% PFA overnight. The embryos are washed in PBS and then transferred to glycerol and ready for imaging.

F. Statistical Analysis

ThermoFisher NanoDrop Plus was used to measure the concentrations of both DNA and RNA in the gene samples. Those concentrations were used to determine the quality of the nucleic acids.

RESULTS

PCR and Gel Electrophoresis

To visualize FGF expression in convict cichlids, the initial step was to build degenerate primers using Tilapia. Given that Tilapia is a closely related cichlid to convict cichlids, it was presumed that they would have a similar genome. The degenerate primers were able to bind to the convict cichlid cDNA, and the segments of DNA were detected for all genes using the polymerase chain reaction. Fig. 5(A,B,C) shows bands observed for FGFr2, FGF8b, ON_FGF17, AC_bFGF, AC_FGFr3, AC_FGFr1, and DR_FGF8a. AC_FGFr1 showed multiple bands, and gel extraction technique was performed in order to isolate the brightest band which contained the highest DNA concentration.

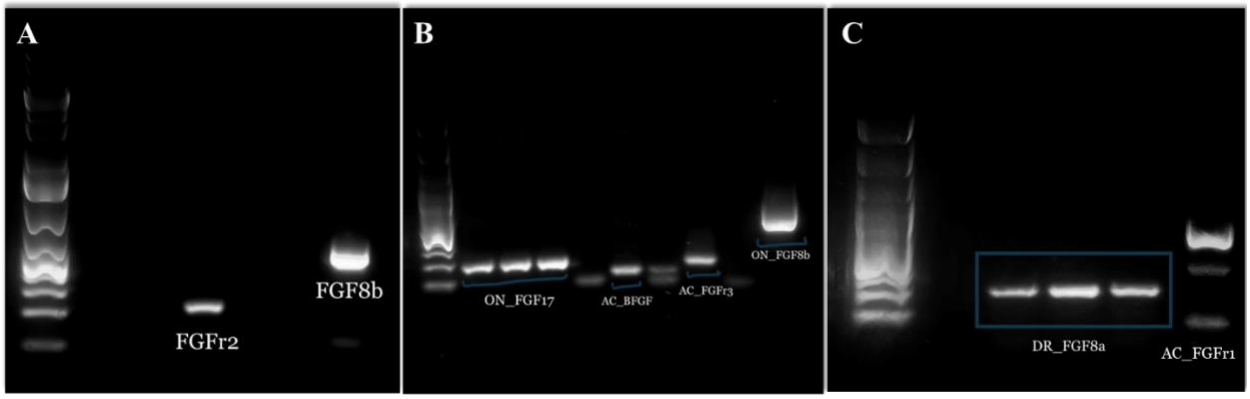

Figure 5. Polymerase-chain reaction gel results. *(A) The gel image shows the results for the primers using the previously sequenced miniprep FGF8b. (B) The gel image shows a successful band for FGF17, βFGF, and FGFr3. (C) The gel image shows three bands for Danio Rerio FGF8a, and one for AC_FGFr1.*

Danio Rerio primers for FGF8a were also ordered, and a PCR was performed using zebrafish cDNA. Fig. 5(C) shows a band PCR for DR_FGF8.

DNA and RNA concentrations

Since the DNA concentrations were low for the FGFr2 and FGF8b minipreps, which were previously made by other students, a Miniprep transformation was done to increase the DNA concentrations for further experiments.

GENES	DNA Concentration (ng/µl)	Peak (260/280)
AC_FGF receptor 1	181.9 ng/µl	1.88
FGF receptor 2_Pd3_Tilapia	177.7 ng/µl	1.90
AC_FGF receptor 3	141 ng/µl	1.90
ON_FGF8b	197.8 ng/µl	1.89
ON_FGF17	155.4 ng/µl	1.88
AC_βFGF	84.5 ng/µl	1.91
DR_FGF8a	120.5 ng/µl	1.83

Table 1. *The DNA concentrations measured for the minipreps made.*

The DNA concentrations acquired were sufficient to create the plasmid digests. Table 1. Shows the DNA concentrations for the miniprep. To visualize the expression of the target gene FGF through In-situ hybridization, the next step was to create riboprobes. The mRNA in the in-situ hybridization can be detected by using Riboprobes, which are segments of RNA. Following the protocol for making the Riboprobes from the clean digest, the concentration measured was supposed to be between 20-100 ng/µl.

GENES (T7 and Sp6 probe)	RNA concentration In 100 µl
FGF receptor 1 (T7)	23.06 ng/µl (low concentration)
FGF receptor 1 (Sp6)	39.1 ng/µl
FGF receptor 2 (T7)	121.4 ng/µl
FGF receptor 2 (Sp6)	21.14 ng/µl
FGF receptor 3 (T7)	38.7 ng/µl (low quality)
FGF receptor 3 (Sp6)	105.2 ng/µl

FGF8b (T7)	44.8 ng/µl
FGF8b (Sp6)	110.4 ng/µl
FGF17 (T7)	6.38 ng/µl (low concentration)
FGF17 (Sp6)	98.6 ng/µl
βFGF (T7)	57.4 ng/µl (low quality)
βFGF (Sp6)	69.2 ng/µl

Table 2. *The RNA concentrations measured for the Riboprobes made.*

Table 2. shows the RNA concentrations that were measured for the Riboprobes created for all the cichlid genes. These concentrations were measured after the dilution with 100 µl of Nuclease free water. The low quality of a sample was determined by the resulting peak and 260/280 wavelength ratio. FGF17, βFGF, and FGF receptor 3 riboprobes had to be made twice, however, the concentration remained low. This could indicate an issue with the plasmid digest. Due to a time constraint, more samples of probe were not created for the genes that had a low RNA concentration. After making the riboprobes, In-situ hybridization for cichlid embryos was performed. This was a four-day experiment in which riboprobes were utilized to visualize the expression of a certain gene in a set of embryos.

Gene Expression Data

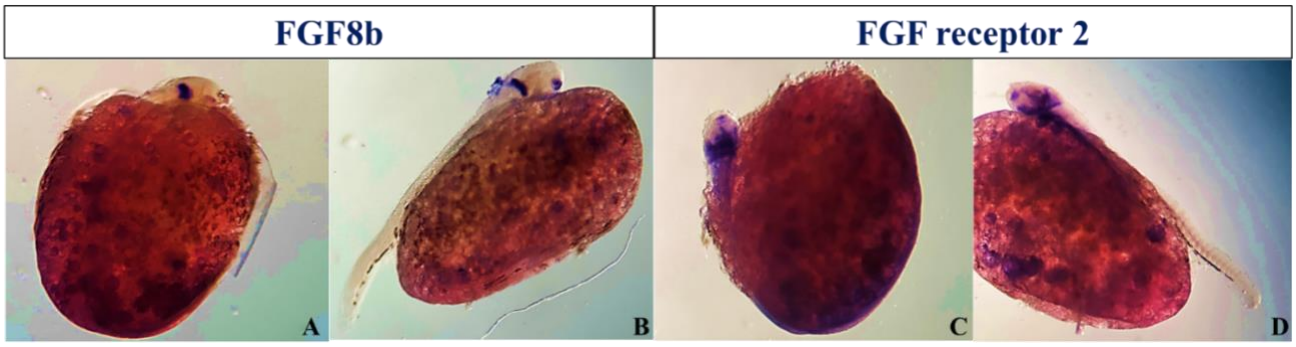

Figure 6: Whole-Mount in Situ Hybridization. *The figure above illustrates the FGF gene expression in convict cichlids. A, C are both at the pharyngula embryonic stage, and B, D are both at the Hatch embryonic stage.*

Only two out of six genes worked. The gene expression was successfully visualized in FGF8b and FGF receptor 2. The gene expression is indicated by the dark purple color on the head of the embryos in Fig. 6-9. For fgf8b, the dark purple line is the Mid brain – hindbrain boundary. The slight purple splash of color on the head is the optic stalk. For Fgfr2, the gene expression is quite prominent in the Central Nervous System, more in forebrain ventricular zone, ventral diencephalon, tegmentum, hindbrain, however, quite weak in the spinal cord.

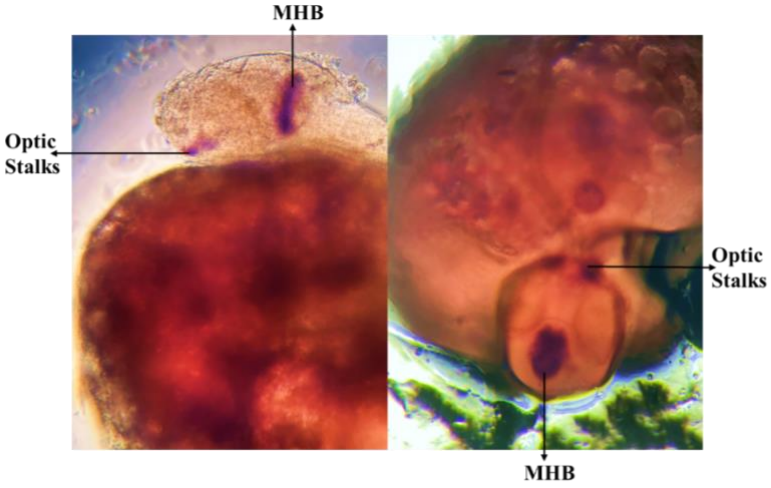

Figure 7: FGF8b Gene Expression in the Hatch stage. *These close ups of the gene expression visualized through in situ-hybridization displays a clear FGF8b gene expression in the optic stalks and outlines the Midbrain-Hindbrain Boundary.*

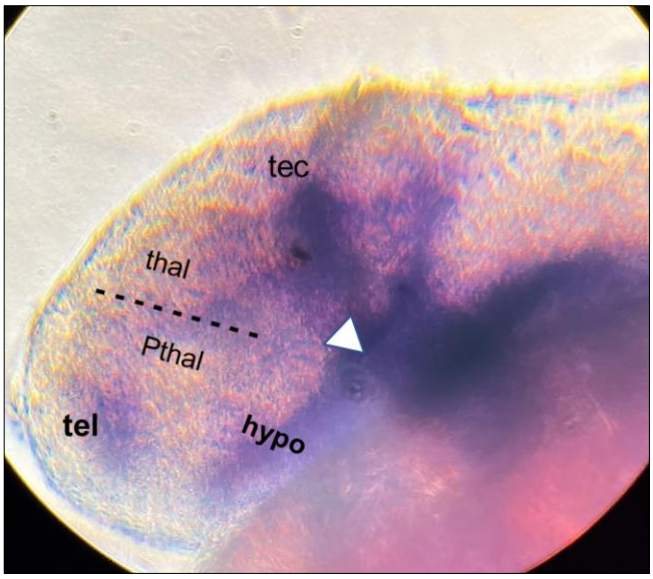

Figure 8: FGF receptor 2 gene expression in the Hatch stage.
White arrowheads mark the position of the MHB; tec, tectum; tel, telencephalon; thal, thalamus; Pthal, prethalamus; hypo, hypothalamus.

Fig. 8. Shows the FGF receptor 2 gene expression in convict cichlids. The areas of the forebrain compartments were not measured according to scale. The anatomical landmarks are only indicative of gene expression pattern. Measurements will have to be taken at developmental phases where compartments are defined by cellular activities using ImageJ software to verify, they are taken from comparable serial sections (Sylvester et al, 2010).

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Fig. 9 shows the difference in the anatomy and gene expression in convict cichlid in comparison to zebrafish.

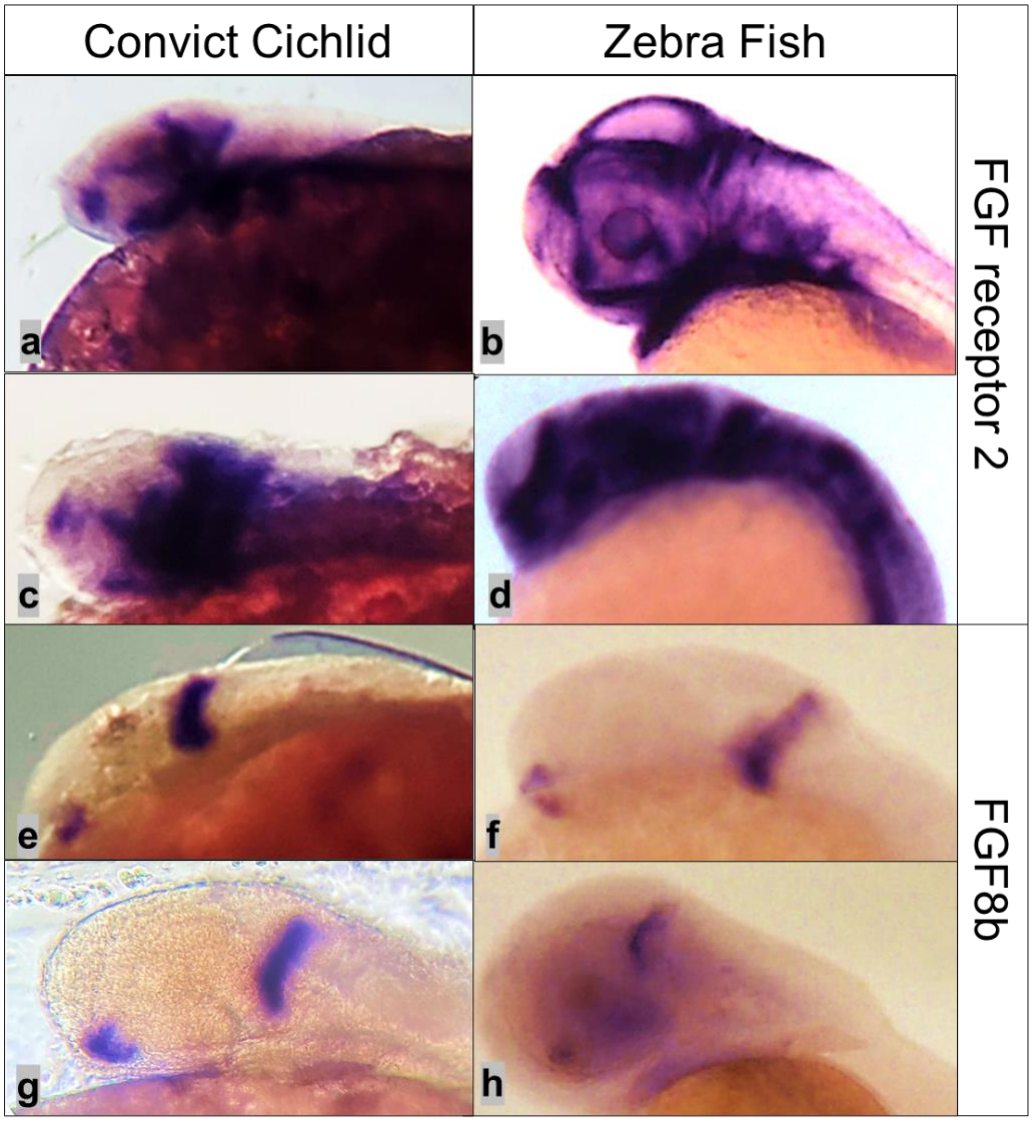

Figure 9: The Difference in Gene expression between the Convict Cichlids and Zebrafish. *The first four images (a-d) represent the FGFr2 gene expression in convict cichlid and Zebrafish, respectively. The images (e-h) portray the FGF8b gene expression in both the convict cichlid and Zebrafish. (a, c, e, and g) depicts the gene expression in the convict cichlids. (b, d, f, and h) depicts the gene expression in the zebrafish.*

Discussion

Cichlid and zebrafish brains develop at different rates, and this comparison is informative for the patterning of the neuroendocrine hypothalamus. Convict cichlids are a monogamous species, whose genome has never been sequenced before. FGF morphogens were used as the experimental component to determine why this is the case, and further study revealed that FGF8 mRNA transcripts were found in the developing telencephalon's midline commissural plate and telencephalic/diencephalic border, as well as the ventral diencephalon (prospective hypothalamus) (Fig. 1A). Furthermore, the primary FGF8 receptor, FGFR1, is expressed in both Rathke's pouch (Fig. 1, B, and D) and a larger area within the ventral diencephalon (Fig. 1, C, and D) (McCabe et al., 2011). In monogamous prairie voles, oxytocin, a neuroendocrine hormone, was shown to cause behavioral distinctions such as monogamy, and a lack of FGF8 signaling was found to cause a decrease in oxytocin neurons. This led to the theory that the monogamous behavior found in convict cichlid fish is due to greater levels of FGF signaling morphogens forming the hypothalamus, which promotes enhanced oxytocin hormone production.

The results of the experiments revealed gene expression for both FGF receptor 2 and FGF8b in convict cichlids. Polymerase chain reaction analysis further revealed that FGF receptor 2 amplifies FGF8b. Detailed examination of gene expression in figures 8-9 suggested that FGF receptor 2 surrounds the mid-brain hindbrain barrier in convict cichlids, particularly during the pharyngula stage. Additionally, the gene expression is detected in the Central Nervous System, specifically in the forebrain ventricular zone, ventral diencephalon, and hindbrain. According to Zfin, FGF receptor 2 gene expression is detectable in the forebrain ventricular zone, ventral diencephalon, tegmentum, and hindbrain during the zebrafish pharyngula stage, but is weak in

the spinal cord, hypo chord, ventral mesenchyme, branchial arches, and lens. This could imply that FGF receptor 2 gene expression is higher in zebrafish. When comparing FGF8b gene expression in zebrafish and convict cichlids, the expression in the midbrain hindbrain boundary and the optic stalk is substantially darker and thicker in convict cichlids. This could imply that convict cichlids have larger quantities of FGF8 proteins building the Midbrain-Hindbrain barrier than zebrafish.

According to the current findings, there is a variation in FGF gene expression between convict cichlids and zebrafish. Another way to visualize the difference in gene expression is to do in situ hybridization on both convict cichlids and zebrafish simultaneously and with similar concentrations of RNA probes. The small decrease in FGF2 gene expression in the convict cichlids could potentially be attributed to a variation in RNA concentration. In addition, another potential gene that can be considered is FGF8a. Transgenic mice with ectopic production of FGF8a and FGF8b in the midbrain showed that FGF8a expanded the midbrain (Lee et al., 1997), whereas FGF8b transformed the midbrain into the cerebellum (Liu et al. 1999). Because both are expressed along the Midbrain-Hindbrain barrier, there may be a discrepancy in the amount of gene expression. This difference in gene expression may also aid in unraveling developmental differences between convict cichlids and zebrafish.

In conclusion, despite significant variations in gene expression, the convict cichlids' monogamous behavior cannot yet be ascribed to greater quantities of FGF signaling morphogens developing the hypothalamus. The visual representation of gene expression shows an increase in FGF8b and a possible decrease in FGF receptor 2. Thus, quantification of expression data by quantitative polymerase chain reaction (qPCR) would be a more effective technique for detecting potential alteration in gene expression.

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