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From Morphogens to Monogamy: the Wnt Pathway's Role in Monogamy

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Abstract

The fish species *Amatitlania nigrofasciata,* convict cichlids, exhibit the behavior of serial monogamy. Dopamine has been linked to the success of pair bonding and monogamy. Dopaminergic (DA) neurons, the receptors of dopamine, are the reason the reward feeling of dopamine is felt by organisms. These neurons are housed in the Ventral Tegmental Area (VTA). The Wnt morphogen, which works through the Wnt pathway, has been previously shown to be a key player in the formation of the midbrain, the area where the VTA resides. This thesis delves into comparing gene expression related to the Wnt morphogen between convict cichlids and the polygamous control, *D.rerio* (zebrafish).

RNA probes were created to target mRNA that codes for specific proteins in the Wnt pathway, *Wnt-1* and *Fzd-1* in convict cichlids and zebrafish. In Situ Hybridization (ISH) using convict cichlid and zebrafish embryos was performed to distinguish gene expression between the two species. Whole-mount images of these embryos were taken to study the placement and concentration of the morphogen. Convict cichlids have been shown to have similar placement to zebrafish with *Wnt-1,* but convict cichlids show heterochrony with the development of *Wnt-1. Fzd-1* placement and concentrations are different between convict cichlids and zebrafish. These first glimpses into the similarities and differences of gene expression may provide insight into the formation of the VTA and DA neurons.

Introduction

Mating Systems

Throughout the animal kingdom, there are two main mating styles. Monogamy occurs when one pair bonds selectively (Young & Wang, 2004). Monogamous pairs can either mate for life, or mate for the breeding season before moving on to pick another mate the next season; this

is serial monogamy (Krasnec, et al., 2012). Conversely, polygamy occurs when organisms do not pair bond and instead mate with as many organisms of the same species as possible (Summers, 2017).

There are benefits and tradeoffs for both styles of societal structures. Monogamy allows for the parental protection and care of the offspring, which, in turn, can increase the number of offspring that survive until adulthood, carrying the parents' genetic material with them (Tumulty, et al., 2014). However, this is mainly beneficial to those who produce offspring rarely throughout their reproductive stage. The biggest trade-off when it comes to monogamy is evolution based. With monogamy, the evolutionary need to spread genetic material as much as an organism can is stilted with the bonding with only one partner, which reduces biological fitness (Klug, 2018). Polygamy, however, focuses much more on spreading an organism's genetic material efficiently. There is no long-term bonding that occurs, and most of the time, the offspring are left to fend for themselves. The goal of polygamous mating is for organisms to have increased fecundity.

Fish especially are large proprietors of the polygamous method (Morris & Rios-Cardenas, 2009; Whiteman & Côté, 2004). For example, the model organism *Danio rerio*, zebrafish, is a strong example of a successful polygamous species. The females can breed all year long, and simply scatter the eggs when they are fertilized, giving no care or protection to their offspring (Spence & Smith, 2006). However, monogamy, particularly serial monogamy, persists in *Amatitlania nigrofasciata*, the convict cichlids (Snekser & Itzkowitz, 2019). Their behavior, in general, is largely fierce and aggressive for both the females and males of the species, especially when it comes to their territory (Barley & Colemon, 2010; Leiser, et al., 2004). Through biparental caretaking, both organisms in the pair bond defend and protect their territory and offspring in clearly designated roles (Itzkowitz, et al., 2001). Amongst thousands of

fish species, convict cichlids are one of the few that expends a great amount of time and energy in the development of their offspring. What is the reasoning for convict cichlids to go against the grain and perform serial monogamy? The answers may reside in the morphogens that influence the development of the brain and the regions within.

The Burgeoning Brain

Many of the same systems in the development of the brain remains the same, even if the animals in question seem to be highly dissimilar (Ghysen, 2003). Homologous genes, signaling molecules, and hormones are rampant in the genomes of species that one would never take to have recent commoner ancestors (Ghysen, 2003; Moroz, et al., 2021).

For the brain, a lot of morphogens are conserved in evolutionary history (Dekanty & Milán, 2011). Morphogens are signaling molecules that work through concentration gradients to determine cell patterning, growth, and maturation during embryonic development. There are many types of morphogens, such as Sonic Hedgehog (Shh), Fibroblast Growth Factor (Fgf) and Wnt/β-catenin (Wnt). These morphogens are important in many ways, but a key facet is their ability to regionalize the brain, determining how exactly parts like the forebrain and midbrain form, two parts of the brain that are key in behavior (Sansom & Livesey, 2009).

All three of these morphogens work through tight regulation, with the help of transcription factors that up- or downregulate the gene expression of these morphogens so that proper development occurs. The placement the morphogens, as shown in Figure 1, are important in understanding where and how exactly these pathways influence brain regionalization and patterning. Fgf works to develop and link the olfactory bulb to the hypothalamus, through the formation of specific neurons that affect mating behaviors. (Chung et al., 2010). Shh works to develop the forebrain and the hypothalamus, forming neural pathways that involve the secretion

and pickup of oxytocin (Grinevich et al,. 2015; Sagai et al., 2019). Finally, Wnt has a role in the development of the dorsal mid-hindbrain region, as well as the hypothalamus (Arenas, 2014; Machluf et al., 2011). All these morphogens are imperative for the development of the brain, and the knockdown or otherwise disruption of one or the other can cause various malformations in the brain, seen especially in the thalamus (Hagemann & Scholpp, 2012). This is important to note and remember; even though this thesis focuses on the Wnt morphogen in particular, it works hand in hand with Shh and Fgf to form and regionalize the brain, and even work to develop same parts of the brain, like the thalamus and hypothalamus.

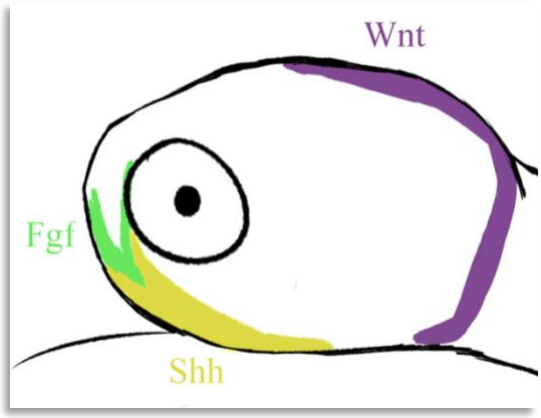

Figure 1. Interactions between morphogens. The three key players in the development of the brain have specific areas in which they reside in (Prakash & Wurst, 2006). Wnt is focused on the posterior, dorsal area of the head. Fgf is focused on the most anterior part of the head, while Shh comes up from the ventral side of the head to meet Fgf.

Canonical Wnt Pathway

The Wnt morphogen works through its multiple pathways (Komiya & Habas, 2008). The canonical Wnt pathway is the main Wnt pathway that is studied when it comes to the differentiation of cells (Mulligan & Cheyette, 2012). The key players in the Wnt pathway are the Wnt extracellular ligand protein, the Frizzled (Fzd) cell membrane-spanning protein receptor, the β-catenin protein, the β-catenin destruction complex, and the transcription factors Lef and Tcf (Komiya & Habas, 2008; Mulligan & Cheyette, 2012). Figure 2 provides a visual representation of the factors involved in the pathway and the process itself. Beginning with the Wnt ligand binding to the Fzd receptor and the LRP (low-density lipoprotein receptor-related protein) co-

receptor, a positive feedback loop begins. Once bound, a disheveled (Dvl) protein phosphorylates and moves to attach to the Fzd receptor in the cytoplasm. Concurrently, an axin protein from the β-catenin destruction complex (βCDC) also moves to attach to the cytoplasmic portion of the LRP5 co-receptor; this disrupts the complex so it cannot destroy the β-catenin in the cell. Now, β-catenin can form a concentration gradient in the cytoplasm of the cell, where it eventually can diffuse into the nucleus and attach to the transcription factors Lef and Tcf, which upregulate the activation of Wnt gene expression. This eventually forms more Wnt proteins which move out of the cell, essentially completing the loop (Komiya & Habas, 2008; Mulligan & Cheyette, 2012).

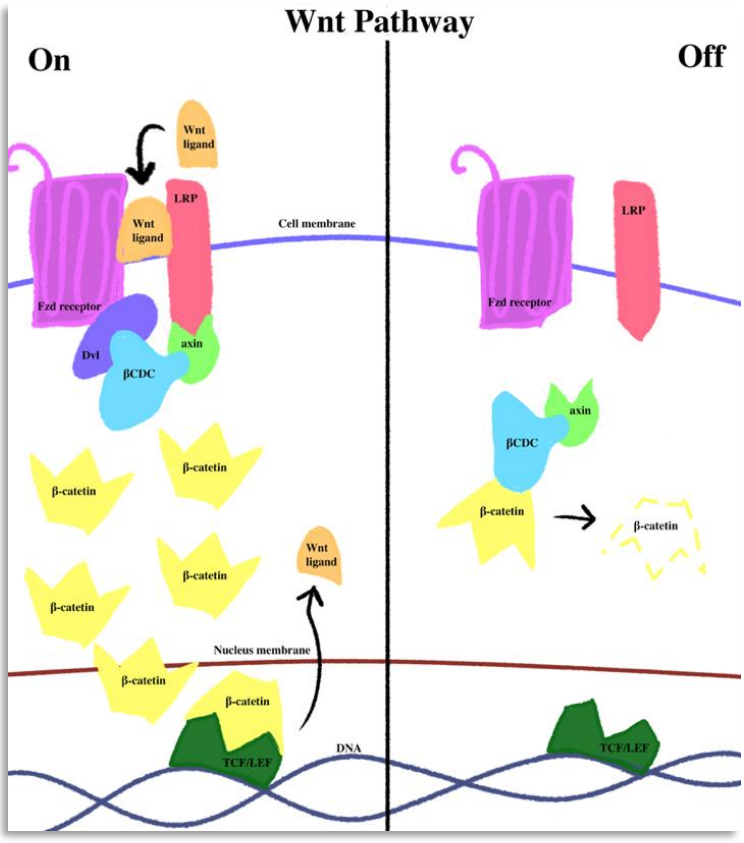

Figure 2. Schematic of the Canonical Wnt Pathway. The end goal of this pathway is the formation of more Wnt ligands to leave the cell and find more cells with the Fzd and LRP receptors. This is done by disabling the multi-protein complex that destroys β-catenin, allowing for the protein to become concentrated enough to diffuse into the nucleus and influence the transcription factors Lef/Tcf.

Both the proliferation and inhibition of this pathway have major effects on embryogenesis, specifically during gastrulation and neurogenesis. The Wnt pathway affects how, when, and where the parts of neural tube forms by controlling progenitor cells formation, regionalization, and maturation (Mulligan & Cheyette, 2012; Prakash & Wurst, 2006; Prakash, et. al, 2006; Wilson & Houart, 2004). Even after the brain becomes fully developed, Wnt can still affect the adult body Unregulated Wnt activity has been linked to cancer, while research has been conducted in using Wnt to cure Parkinson's disease (Marchetti et al., 2020; Zhang & Wang, 2020). Overall, the Wnt pathway is important facet of the body, from embryogenesis to adulthood.

Wnt's Role in the Midbrain

As seen in Figure 1, the position of Wnt, specifically *Wnt-1,* is on the posterior side of the head, curling down toward the ventral side. *Wnt-1* gene expression, in the rodent, has been located throughout the midbrain of the embryo, from the caudal side dropping toward the rostral area and encroaching along the ventral middle of the midbrain (Prakash & Wurst, 2006). Experimentation on zebrafish embryos has found that Wnt-1 resides around the same areas as seen with the rodent embryo (Lekven, et al., 2003; Molven, et al., 1991). This showcases the homologous nature of *Wnt-1* between multiple species. *Wnt-1* surrounds a very important cluster of neurons that facilitate the feeling of pleasure and reward. This hub is called the Ventral Tegmental Area (VTA).

The VTA area in the midbrain plays a big role when it comes to pleasure, motivation, and reward in vertebrate animals (Bouarab, et al., 2019). Since this is the area that hosts mature dopaminergic (DA) neurons, it is the main hub of the mesolimbic pathway, that offshoots dopamine to the nucleus accumbens and the amygdala (Van den Heuvel & Pasterkamp, 2008).

This circuit is the reason that people feel a rewarding effect when it comes to things like sex and drugs (Pariyadath, et al., 2016; Balfour et al., 2004). This is due to the DA neuron hub that is nestled in the VTA (Figure 3). The neurons are the conduits to an organism feeling the rush of dopamine that floods the brain when doing something particularly rewarding, which leads them to wanting to repeat the activity (Pariyadath et al., 2016). The more DA neurons that are a part of the VTA, the stronger effect the dopamine will have on the organism (Edwards & Self, 2006). These DA neurons begin as progenitor cells from the ectoderm of the gastrula before they regionalize to the VTA and mature during neurogenesis (Wang et al., 2020). The progenitor cells can grow and divide until they mature enough to become post-mitotic progenitor cells (Russek-Blum et al., 2008). At this point, the cells cannot divide anymore, and have a fixed population. The last stage of DA neuron development involves the neurons becoming functional adult neurons. *Wnt-1* has been linked to the cell fate and determination of these neurons, so it's able to come in and essentially order these progenitor cells to become functional DA neurons (Wang et al., 2020; Castelo-Branco et al., 2003).

Wnt, along with the help of Shh and Fgf morphogens, which all surround the midbrain, induce DA progenitors to form in the ventral midbrain, close to where the VTA would be (Prakash & Wurst, 2006). Wnt is needed, in some part, for the maturation and differentiation of these DA progenitor neurons in the midbrain (Castelo-Branco et al., 2003).

Wnt's Role in the Forebrain Region and the Hypothalamus

The VTA of the brain is not the only important part for the development of DA neurons. As mentioned before, the VTA is the hub of neurons that work as part of the mesolimbic pathway. As the neurons are activated with a rush of dopamine, that biochemical signal is sent through this pathway into ventral areas of the brain (Yamamoto & Vernier, 2011). The

hypothalamus is a critical region that influences an organism's behavior, including pair ponding and sexual behavior (Machluf et al., 2011). Other DA neurons also project into the ventral diencephalon of the brain, which functions as part of the limbic system, meaning it's also important in the behavior and emotional responses in organisms (Michael-Titus et al., 2010; Rajmohan & Mohandas, 2007). Due to this, it's important to look at the expression that occurs throughout the brain, not just the midbrain region (Figure 3).

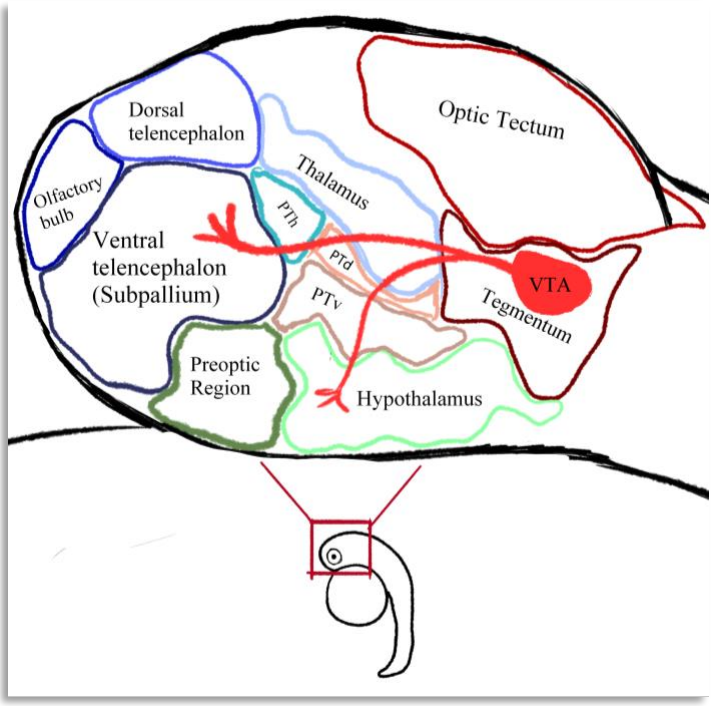

Figure 3. **Brain regions in the zebrafish.** Sagittal diagram of the different brain regions, anterior on the left and dorsal pointing up (Ronneberger et al., 2012). The VTA is formed in the tegmentum of the midbrain (Cai & Tong, 2022). The VTA's DA neurons spread throughout the brain, but the most notable reaches are into the hypothalamus and the ventral telencephalon; the DA neurons are also present in the PTd (dorsal posterior tuberculum) and the PTv (ventral posterior tuberculum), helping with the relay system of information to the sub pallium and diencephalon (Machluf et al., 2011; Vernier & Willimann, 2009).

Stage Dependence of the Wnt Morphogen

Evidence has shown that proliferation and differentiation of the mid-/hindbrain is influenced by the Wnt morphogen. However, another side that needs to be considered is the stages of embryonic development when looking at the influence of the Wnt morphogen.

For example, during gastrulation, research has been shown that the Wnt morphogen has a negative effect on DA progenitor neurons, seen through the downregulation of Wnt in zebrafish embryos and the increase of the progenitor pool compared to the wild type (Russek-Blum et al., 2008). This leads to, overall, a higher amount of mature DA neurons during later stages. However, other research posited that older zebrafish embryos, past the gastrulation stage, have higher degree of mature DA neurons when the *Wnt-8a* gene is overexpressed (Westphal et al., 2022). The overexpression of *Wnt-8a* during the gastrulation stage (10 hpf) can also been seen to cause deformities to the anterior portion of the embryo, showcasing that a possible disruption of the Wnt anterior-posterior axis formation may have occurred (Westphal et al., 2022). Figure 4 depicts the rationale of stage dependance between these two studies. This evidence provides another angle to look at, as the expression may shift as the embryos grow through development, specifically those noted in Figure 5.

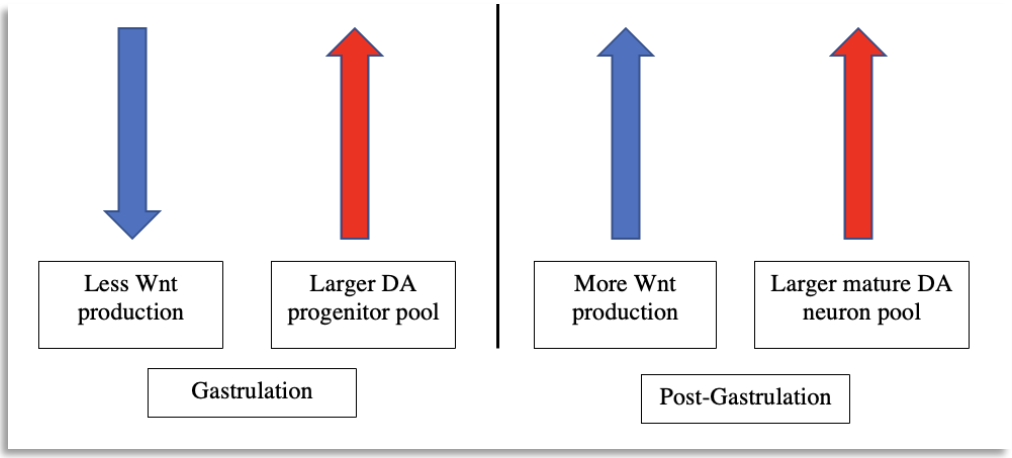

Figure 4. Stage dependance of the Wnt morphogen. Previous studies have shown that during gastrulation, Wnt antagonizes the development of DA progenitors, but has the opposite affect on maturing DA neurons after gastrulation has finished (Russek-Blum et al., 2008; Westphal et al., 2022). Another side of this comes in the form of the timing during post gastrulation, where the effect of Wnt on DA neurons loses it's potency as time passes (Westphal et al., 2022).

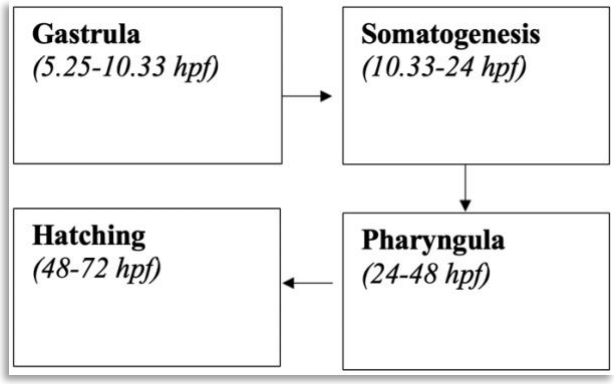

Figure 5. Critical stages of development in the zebrafish embryo (Kimmel, et al., 1995). Note that the hours post fertilization (hpf) are relevant only to zebrafish, not convict cichlids.

This is especially crucial since the production and presence of Wnt is much like using a double-edged sword to the newly formed progenitor cells. On the one hand, the Wnt morphogen's job is to specify cells, leading them to develop the necessary parts that they will later need as functional cells (Bhavanasi & Klein, 2016). Since the progenitor cells can turn into any kind of cell, much like the ability of stem cells, Wnt's affect, through signal cascades and concentration gradients, would be setting them up to become mature DA neurons. This is great, until the other shoe drops and these cells become functional before they can divide enough. The presence of Wnt not only specifies but matures these progenitors; this is Wnt's second function (Salinas, 2012). This can lead to less division in the progenitors, especially if there is a lot of Wnt in the area, overall diminishing the amount of mature DA neurons in the brain. So, the timing of Wnt during development needs to be heavily regulated, so there can be some division of the progenitor pool, but still achieve the neural connections that need to be in place for proper communication through hormone signals like dopamine.

Marriage of Ideas

As mentioned previously, the fish species convict cichlids exhibit a serial monogamist mating style (Snekser & Itzkowitz, 2019). Looking into the developmental pathways and embryogenesis of previous model organisms like rodents and zebrafish, the Wnt morphogen, specifically *Wnt-1*, may have something to do with the rare mating behavior of convict cichlids. Specifically focusing on the VTA and midbrain, *Wnt-1* has been shown to proliferate and mature the DA neurons that reside there during gastrulation and neurogenesis in other vertebrate organisms (Castelo-Branco, 2003; Prakash & Wurst, 2006). The DA neurons are what facilitate the uptake of dopamine that floods the brain during mating (Balfour et al., 2003). Another key part of the puzzle is looking for Fzd expression. This is important because the expression that would be visualized correlates with the number of cells that are able to perform the Wnt pathway. Visualizing the expression on multiple embryos that were fixed during different times in development is also crucial, as previous research has noted that the Wnt morphogen seems to have differing impacts on the development of DA neurons at different stages when it comes to DA neurons.

By looking for the *Wnt-1* and *Fzd-1* gene expression in convict cichlids and zebrafish, any differences perceived in the visualization of the expression may lead to a larger increase in the DA neuron pool for the convict cichlids, leading to a fish that feels more pleasure in mating and so decides to stick with the partner it mated with. Different stages of development were also studied to view how *Wnt-1* and *Fzd-1* expression changes as the embryo grows. This gene expression difference may be the clue that can shed some light on why organisms like convict cichlids perform serial monogamy.

Methods and Materials

Probe Synthesis

Forward and reverse primers were ordered for *Wnt-1* and *Fzd-1* genes for both convict cichlids and zebrafish *(*DR*),* as shown in Table 1. Since *A. nigrofasciata* has not been extensively genome sequenced, the relative *Maylandia zebra's* (MZ) genome was used for the primers. Amplification of cDNA using the forward and reverse primers was accomplished through polymerase chain reaction (PCR) tests. Reamplification allowed for the further amplification of the genetic sequence needed. Ligation was performed using pGEM T-easy vector to clone the sequence and insert into the plasmid. This plasmid was then inserted into JM109 *E. coli* bacteria using the process of transformation. After successful growth, the colonies were spun down and the resulting pellet was washed to form the miniprep. The miniprep was digested using both *Sac-1* and *Nco-1* enzymes, separately. These digests were used to synthesize the probes using T7 and Sp6 polymerases, respectively, to make T7 and Sp6 probes. Probes were stabilized with hybridization buffer.

In Situ Hybridization

Embryos were digested with Proteinase K, at varying dilutions and time depending on the age of the embryos. Embryos incubated with the designated probes for around 16-18 hours. Anti-DIG antibody was used, 1:3000 dilution for cichlid embryos and 1:5000 dilution for zebrafish embryos. Color reaction was done using 1-step NBT/BCIP. Embryos were refixed after the color reaction was completed and were stored in a glycerol and phosphate-buffered saline (PBS) solution.

Species	Gene	Forward primer sequence	Reverse primer sequence	Enzyme
MZ	*Wnt-1*	5’ CCA TAA AGG AGT GCA AGT GG	5’ CCC TTG TTG GCA TAA ACA AC	*Sac1*
MZ	*Fzd-1*	5’ TCA CAA ATT GAC CGA GGA TT	5’ ATG GTG TAG CAG CCA GAA AG	*Sac1*
DR	*Wnt-1*	5’ ACG CTA TCT GAC CAA CTG C	5’ TGT AAG CCC TCC CTA TTT ACC ACC	*Nco1*
DR	*Fzd-1*	5’ ATC ATA TTC CTG TCC GGC TG	5’ AGG AAT TGA GCG TCT TTC CA	*Sac1*

Table 1. Gene sequences used for probes. Species refers to which fish species was used for the primer sequence. The enzyme refers to which digest worked, leading to the probe with the antisense.

Results

Embryos that were fixed at multiple stages were used, from late somatogenesis to hatching; these stages are all post gastrulation. Visualization has been procured for all these stages for the convict cichlids, and both *Wnt-1* and *Fzd-1* expression has been visualized for all three stages. For zebrafish, the results of ISH were not as constant. There is no data for the expression of *Wnt-1* or *Fzd-1* for the late somatogenesis stage. There is no data of *Fzd-1* expression for the pharyngula stage. The hatching stage is the only stage that has expression for both species, as well as for both genes that are being looked at. Below are various figures depicting the expression at different angles. Please note that the images may differ in quality, as two different microscopes were used to take the pictures.

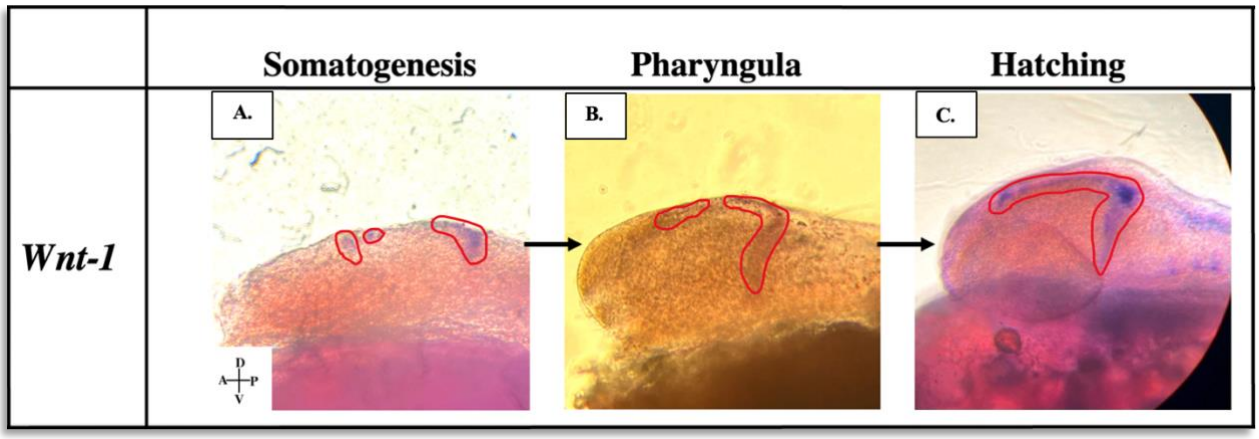

Figure 6. Development of *Wnt-1* in convict cichlids. Whole mount visualization of *Wnt-1* expression in convict cichlid embryos, from somatogenesis to hatching. View is sagittal, with the anterior pointed left and dorsal pointed up. Expression is circled in red for clarity.

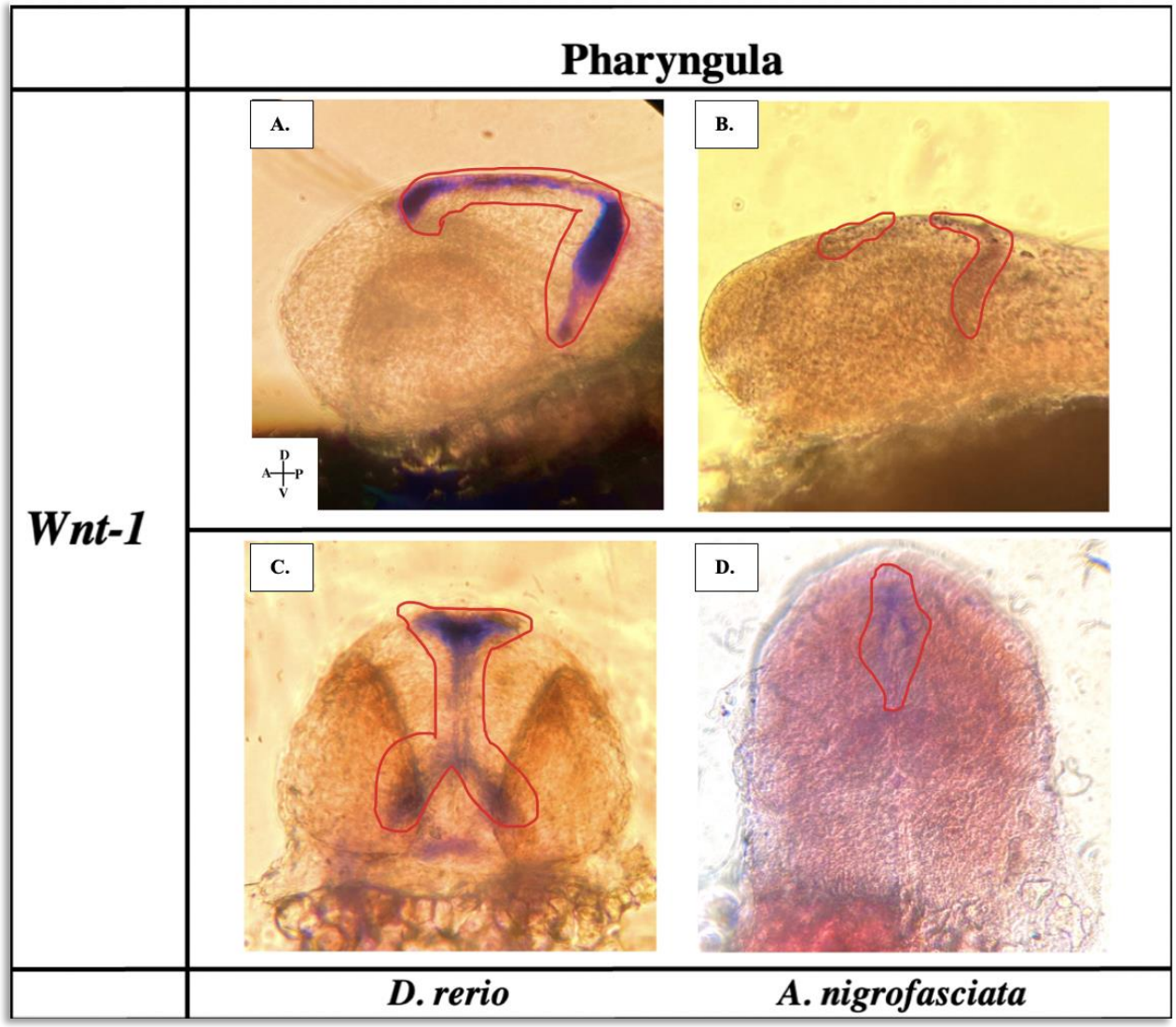

Figure 7. Comparison *Wnt-1* expression during the pharyngula stage between zebrafish and convict cichlids. 7A and 7C depict the zebrafish embryo at different angles, with 7A showcasing the sagittal view, and 7C showing the embryo from the ventral side. 7B and 7D visualize two separate embryos, with 7B depicting sagittal and 7D showing the embryo from the dorsal side. Expression for the embryos are encircled in red for clarity.

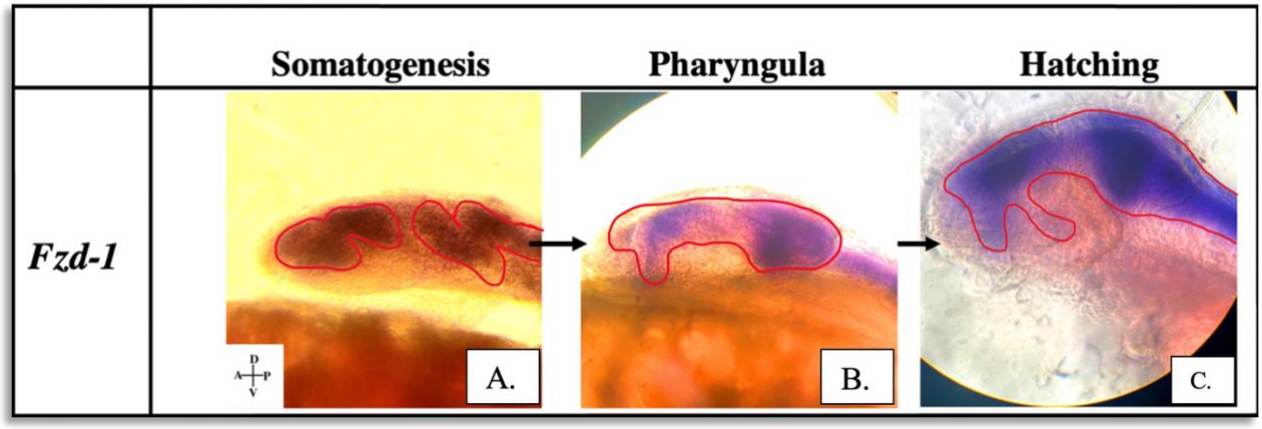

Figure 8. Development of *Fzd-1* expression in convict cichlids. Whole mount visualization of *Fzd-1* expression in convict cichlids, developing from somatogenesis to hatching. Sagittal view, with anterior pointing to the left and dorsal pointing up. Expression is circled red for clarity.

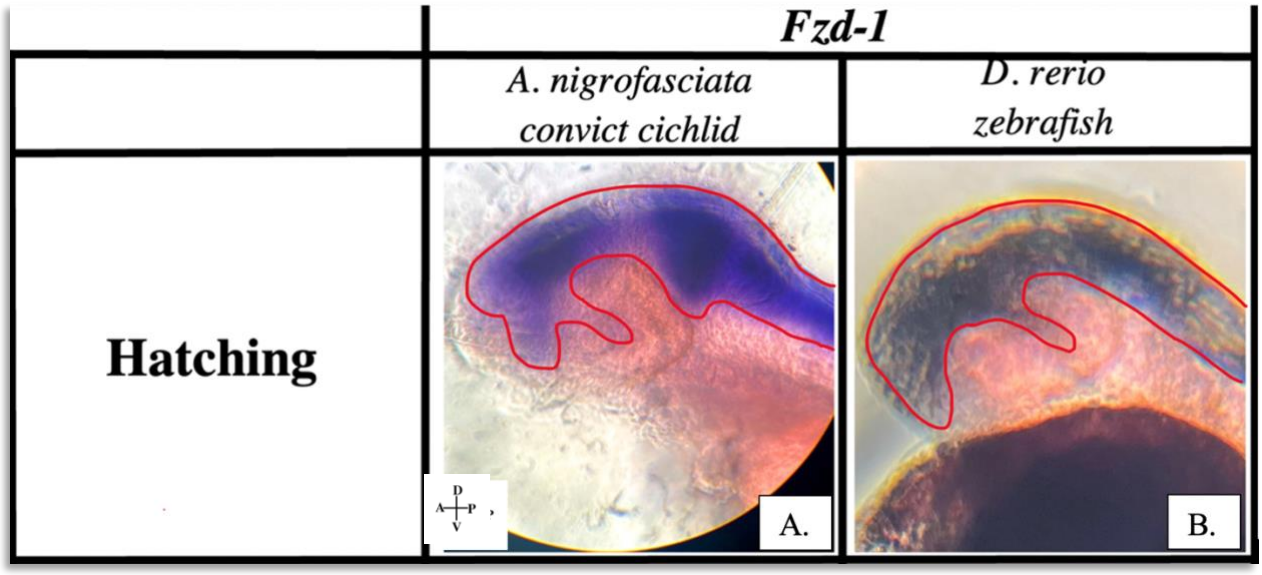

Figure 9. Comparison of *Fzd-1* expression between convict cichlids and zebrafish. Whole mount visualization of *Fzd-1* expression in convict cichlids and zebrafish at the hatching stage, depicting the similarities and differences. Sagittal view, with anterior pointing to the left and dorsal pointing up. Expression is circled red for clarity.

Discussion

Wnt-1 expression

The results for *Wnt-1* were very confusing, in the beginning. The expected results were there to be lots of expression, especially during the pharyngula stage. This is due to previous research conducted, showing that overexpression of *Wnt-8a* in zebrafish, between 15 and 25 hpf (roughly the pharyngula stage), resulted in larger DA neuron clumps in the transgenic zebrafish later on (Westphal et al., 2022). However, as seen in Figure 6 , the development of *Wnt-1* moves at a snail's pace. There is barely any expression in both the somatogenesis and pharyngula stages. It's only in the hatching stage, well passed the golden hours of development seen in the Westphal research, that *Wnt-1* has the characteristic expression in placement, as well as decent concentration.

This expression disparity becomes further interesting when the zebrafish results are considered. As seen in Figure 7, by the pharyngula stage in development, the visualization of *Wnt-1* for the zebrafish embryo shows strong *Wnt-1* expression. The dorsal top and midline areas of the zebrafish have plenty of expression. Moving down into the midbrain area of the embryo, the expression continues to be strong. However, the same stage for convict cichlids shows very weak expression of the gene. The dorsal side of the brain the characteristic pattern of *Wnt-1* expression, but much less development. Also, there does seem to be expression on the mid-/hindbrain boundary, but there is not much concentrated expression at all. Seen side-by-side, the convict cichlid does not have nearly the amount of expression that zebrafish do.

Looking at both the slow development of *Wnt-1* anteriorly, as seen in Figure 6 as well as the difference of expression between zebrafish and convict cichlids at the same stage in Figure 7**,** it can be interpreted that there is a higher degree of regulation when it comes to *Wnt-1* expression in convict cichlids. This time-dependent regulation harkens back to the previous study mentioned briefly in the introduction: antagonizing *Wnt-8a* with Dkk1 during early gastrulation led to a larger DA neuron population in the transgenic zebrafish (Russek-Blum et al., 2008). This is probably due to Wnt's twofold effect on progenitor cells: specifying and maturing cells. Through specifying the cells, Wnt's job at turning them on to become functional DA neurons is also in effect; you cannot have one without the other. At this point, there can be no more division in the cells, so the functional DA neuron pool becomes fixed at the number they matured at. This is problematic if Wnt is expressed earlier, as there's no time for the cells to divide as much as they could.

However, this study artificially decreased Wnt activity. The repression of Wnt seen here in convict cichlids must come from a natural antagonist. The most plausible answer might be one

of the morphogens referenced earlier that surround the mid-/hindbrain region as well (Prakash & Wurst, 2006) In particular, the Shh morphogen may have something to do with the *Wnt-1* repression seen in convict cichlids; Shh and Wnt are naturally opposing forces, and antagonize one another (Ding & Wang, 2017). Shh may be the natural force that quells the expression of *Wnt-1* earlier in development.

With these ideas, it's clear that Wnt expression needs to be carefully used and delegated (Figure 10). As seen in the convict cichlids, *Wnt-1* is highly regulated in the earlier stages. This regulation stifles Wnt's ability to mature the progenitor cells. The progenitor cells, in turn, will have more time to grow and divide. Later, during the hatching stage, the strict regulation is decreased to allow for more Wnt expression, which can then facilitate the maturation of the progenitor pool. Since there was more time for the progenitor pool to grow, the specification and maturation of the cells could lead to a higher count of DA neurons in convict cichlids, when compared to zebrafish. This proposition would fall under the idea of heterochrony, the evo-devo concept of a change in the timing in developmental processes (Keyte & Smith, 2014).

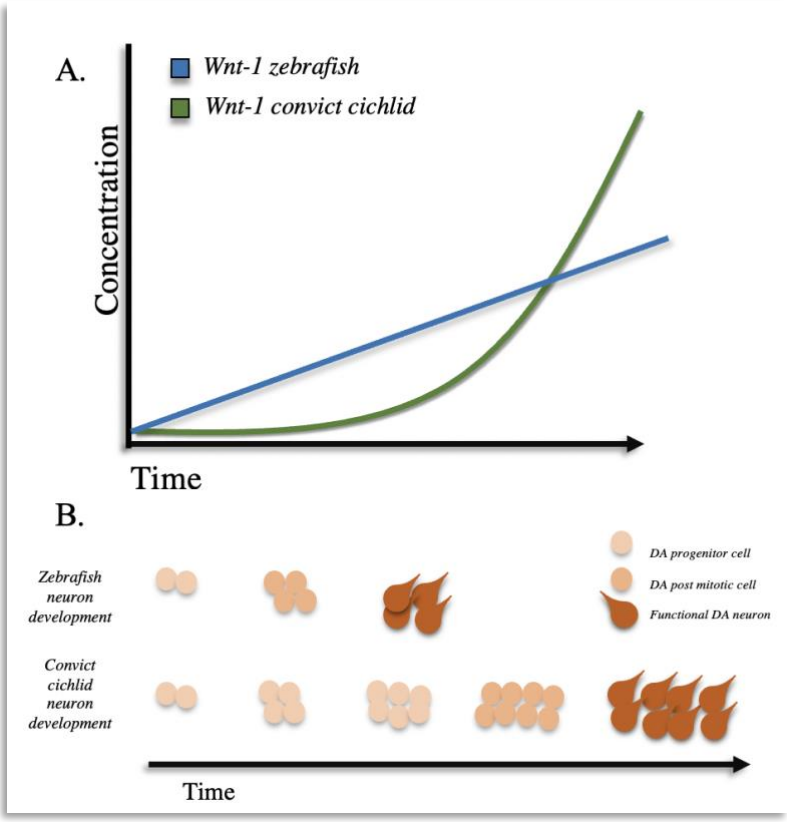

Figure 10. Schematic explanation of the *Wnt-1* gene expression in convict cichlids. 10A depicts the heterochrony that has been visualized in the convict cichlid embryos. While zebrafish may have a linear expression, increasing as the embryo develops, convict cichlids may have their *Wnt-1* expression repressed for a longer period of time. The proposed effect of this delay in expression is seen in 7B, where progenitor cells are given a longer time to replicate, leading to a bigger pool that can than become specialized and functional through the help of *Wnt-1*.

Fzd-1 expression

The development of *Fzd-1* is interesting to see in convict cichlids (Figure 8). In the somatogenesis stage, it is already prioritizing the midbrain areas as well as the sub pallium and hypothalamic regions. This can be seen in the two heaviest parts of the expression in those areas.

In the pharyngula stage, the expression has grown throughout the whole midbrain area of the embryo. It's also still expressed in the sub pallium area of the forebrain. In the hatching stage, the two pockets of expression can still be seen, with the expression comprising the midbrain having shrunk, yet still clearly extending toward the midbrain of the embryo, which encompasses the posterior tuberculum.

While there is only data for the hatching stage of *Fzd-1,* information can still be gathered (Figure 9B). The zebrafish *Fzd-1* expression stays above on the dorsal side of the embryo, where *Wnt-1* is also expressed, and does not show any expression toward the midbrain of the embryo. This may signal that any maturation of neurons that would happen has already been completed, like it is depicted in Figure 10B. From what can be seen in the way expression changes in convict cichlids, the zebrafish *Fzd-1* expression may show up more ventrally earlier in development, but more research in those stages needs to be done to confirm this idea. In Figure 9, the comparison between the two species and their *Fzd-1* expression, shows that convict cichlids have more expression ventrally, near the mid-/hindbrain region, and both have expression on the dorsal side of the embryo.

The data suggests that a lot of cells are secreting the mRNA for the *Fzd-1* protein in convict cichlids. This means a lot more cells that can receive the Wnt ligand are present throughout development. For the midbrain region, the *Fzd-1* expression moves ventrally far more in convict cichlids than in the data collected for zebrafish. This is especially interesting to note, as the VTA is formed and comprises this exact area. This shows that a lot of cells that can interact with Wnt are clustered around the VTA for a longer period, perhaps aiding in the maturation and functionalization of the DA neurons. This aligns well with the proposition depicted in Figure 10A. The longer presence of *Fzd-1* expression in convict cichlids may allow

for the slower development of *Wnt-1* to occur, as there will still be cells responding the Wnt in the area, as opposed to in zebrafish, where the *Fzd-1* expression moves much more rapidly to the dorsal part of the brain.

Both the convict cichlid and zebrafish *Fzd-1* expression can be seen in the sub pallium/ hypothalamic region of the forebrain. This area, as you remember, is where the neurons that form in the VTA project to. This means that there may be some regulation and maturation of DA neurons in this region as well. Another area where cells are expressing *Fzd-1* that is key is the posterior tuberculum; since this is the area where the first DA neurons functionalize, the area around it becomes less expressive as the embryos develop.

The lack of expression for *Wnt-1* in the same areas that *Fzd-1* expression may seem counterintuitive, especially near the forebrain area. However, the *Fzd-1* protein may accept Wnt ligands that are not only formed by *Wnt-1*, but by other Wnt genes as well. A further look into the development of other Wnt genes is imperative to find any correlations there.

Further research

As these results are very preliminary, many more trials of ISH need to be performed. Replication of the data that has been gathered needs to be achieved, along with new data looking at a narrower range of developmental stages between the two species. While the stages of development shown here follow each other in the broad sense, each stage has a wide frame of time in which many things may occur. It's imperative to explore how the expression grows and changes within the stages as well. More research into the expression of *Wnt-1,* particularly as the embryo develops through the pharyngula and hatching stages should be studied, to see when exactly the brain increases it's *Wnt-1* gene expression. Overall, more ISH needs to be done to gather a larger data set for the *Wnt-1* expression in both convict cichlids and zebrafish. This will

garner a better representation of the expression; the data shown here will also become stronger with the more trials run for the same stages.

Another step would be to visualize data of other Wnt genes to find any correlation between them and the large concentration seen of *Fzd-1* in convict cichlids. Some Wnt genes of particular interest would be *Wnt-3a* and *Wnt-5,* as they have influence on the maturation of DA neurons in the midbrain, and *Wnt-8b,* as it's been noted to function in the development of the forebrain and mid-/hindbrain areas. (Arenas, 2014; Brafman & Willert, 2017).

Another goal that would further this research would be to quantify the expression of *Wnt-1* in convict cichlids and zebrafish. This would be the most helpful at the stage of development where the intersection between expression would be seen (as shown in Figure 10A). This will allow us to find out if the proposed inversion of expression is accurate, and when exactly it may occur. This could be done using qPCR techniques.

Manipulating the levels of expression may provide the best way to see if the Wnt morphogen has any discernable effects on the pair bonding behavior seen in convict cichlids. This may be done by bathing embryos in a Wnt agonist, or even a Shh antagonist, to expand the expression of *Wnt-1* to see if stabilizing the expression (much like the proposed rate of expression in zebrafish, shown in Figure 10A) may decrease their ability to pair bond as adults.

Conclusion

Wnt-1 has been shown to exhibit the principle of heterochrony in convict cichlids, where the delay in it's development may lead to more functional DA neurons in the long run. *Fzd-1* expression also differs between convict cichlids and zebrafish, where convict cichlids have expression that moves farther ventrally toward the VTA area of the midbrain. Both pieces of evidence indicate that a difference in neuron growth and development may be at play.

The results of the gene expression seen here can be thought of as a snowball effect. While minute, the change of timing shown in the Wnt morphogen expression between the two species may cause a differing concentration of DA neurons later. This in turn can lead into the differences in behavior seen by the two fish species. The proposed increase in neurons that facilitate the movement of dopamine through the brain in convict cichlids could be one of the reasons that the fish showcases monogamist behaviors. However, the effects of other morphogens, such as Shh and Fgf, cannot be ignored. Their responsibilities in the brain, coupled with the up and down regulation of these and other morphogens through transcription factors, can be combined with the efforts of Wnt, explained here, to lead to the complex social behaviors seen in convict cichlids, and perhaps other monogamist species, like humans.

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