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Evaluating Environmental Enrichment as a Preventative Treatment in a Zebrafish (Danio rerio) Model of Alzheimer's Disease

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Evaluating Environmental Enrichment as a Preventative Treatment in a

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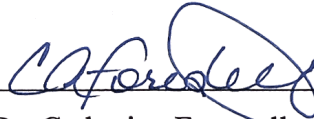
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ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that leads to several cognitive deficits, including impairments in spatial memory (Alzheimer's Association, "What is Alzheimer's Disease?," 2024). This is thought to occur due to atrophy in the hippocampus and cholinergic system (Ferreira-Vieira et al., 2016). Acetylcholine receptor antagonists, such as scopolamine, can mimic the effects of AD by decreasing acetylcholine activity at muscarinic receptors in the hippocampus. Scopolamine is an antiemetic that is FDA approved to treat certain kinds of nausea, but it has become a popular pharmacological model for studying the cognitive impairments associated with AD (Bajo et al., 2015). While efforts are being made to find new treatments and drug therapies for AD, it seems that little progress will be made until its complex etiology and pathophysiology is understood. In the meantime, researchers have started to look at preventative treatments, such as enriching the environment, to minimize the effects of known risk factors (Leshner et al., 2017). The present study analyzed whether zebrafish (*Danio rerio*) with cognitive impairments (induced by scopolamine) are able to demonstrate spatial memory after being raised in an enriched environment prior to drug administration. This was a two-by-two study with the variables being environment (enriched, impoverished) and drug (scopolamine, water). The fish were tested in a Y-maze that assessed spatial alternation, response alternation, and response repetition. The results indicated that there were no differences between groups, and that the zebrafish did not show spatial or response alternation, demonstrating that spatial memory is not in use. The zebrafish showed a slight preference towards response repetition, which is another indication towards a lack of spatial memory.

INTRODUCTION

This thesis serves to evaluate environmental enrichment as a preventative treatment for Alzheimer's disease in a zebrafish (*Danio rerio*) model. This section first presents a brief overview of the relevant topics, followed by a more detailed description of Alzheimer's disease, environmental enrichment, zebrafish, spatial memory, continuous spontaneous alternation behavior in the Y-maze, and scopolamine.

Dementia, one of the most common cognitive disorders in old age, is characterized as a loss of cognitive abilities that cause significant problems in daily activities and functioning. The most common type of dementia is Alzheimer's disease (AD) (CDC, 2020). AD is a progressive neurodegenerative disease that commonly leads to impairments in spatial and episodic memory (Alzheimer's Association, "What is Alzheimer's Disease?," 2024; National Institute on Aging, 2022, 2024). It is estimated that over 55 million people worldwide are currently suffering from AD, and if there are no breakthroughs or new developments, this number will increase to 152 million by 2050 (BrightFocus Foundation, 2024). One of the theories behind this predicted rise in AD is the increase in life expectancy. Since age is thought to be the main risk factor for AD, it makes sense that as people are living longer and therefore increasing the older population, there is also an increase in the number of people affected by AD (Alzheimer's Association, "Causes and Risk Factors for Alzheimer's Disease," 2024).

As research with Alzheimer's disease has progressed, there has been more interest in discovering potential preventative treatments that can be implemented (Leshner et al., 2017). Specifically, environmental enrichment is thought to help mitigate cognitive impairments and slow the progression of AD through strengthening and increasing synaptic activity (Jankowsky et al., 2005). The impact of environmental enrichment can be studied by seeing if there is an

improvement in spatial memory in populations that are exposed to an enriched environment prior to the onset of symptoms. Impairments in spatial memory are associated with AD, as Alzheimer's disease is thought to cause atrophy in the hippocampus and cholinergic system (Ferreira-Vieira et al., 2016; Haam & Yakel, 2018; Maurer & Williams, 2017). When these are damaged, problems with spatial navigation and orientation are observed (Zhu et al., 2017). Therefore, by administering a cholinergic antagonist such as scopolamine, the decline in spatial memory due to AD can be modeled in animals (Bortolotto et al., 2015).

The zebrafish (*Danio rerio*) has been an increasingly popular model organism based on their neuroanatomical and neurophysiological similarities to humans and sharing roughly 70% of the same genes (Burgess & Burton, 2023; Friedrich et al., 2013). This opens the door to endless possibilities for clinical applications and breakthroughs (Veldman & Lin, 2008). Zebrafish have been used to study spatial learning and memory (Cognato et al., 2012; Reemst et al., 2023), and may therefore be used to study AD. They are also capable of absorbing drugs through their skin and gills, which makes drug administration extremely efficient (Friedrich et al., 2013; Morikane et al., 2020). This study aims to evaluate whether environmental enrichment is an effective preventative treatment in a zebrafish model of Alzheimer's disease, using the cholinergic antagonist scopolamine to impair spatial memory in a way that mimics the effect from AD.

Alzheimer's Disease

Alzheimer's disease, as stated previously, is a progressive neurodegenerative disease that commonly leads to impairments in spatial and episodic memory (Alzheimer's Association, "What is Alzheimer's Disease?," 2024; Du et al., 2018; Mayo Clinic, 2024; National Institute on Aging, 2022). Neurodegenerative diseases are chronic conditions that "occur when nerve cells in

the brain or peripheral nervous system lose function over time and ultimately die" (National Institute of Environmental Health Sciences, 2022). Neurodegenerative diseases are thought to be incurable due to this irreversible apoptosis and atrophy it inflicts on different areas of the brain (Cleveland Clinic, 2023; Ferreira-Vieira et al., 2016). Therefore, the current goal for treating neurodegenerative diseases lies not in reversing the effects, but in slowing down the progression of the disease to maximize quality of life or finding ways to minimize risk of onset (Shusharina et al., 2023). Modern research efforts for treating AD are directed towards finding reliable biomarkers, understanding underlying biological mechanisms, and developing diagnostic measures (Du et al., 2018).

There are several obstacles that are limiting the progress of research surrounding Alzheimer's disease (Frozza et al., 2018). This is mainly due to the complexity of the etiology and pathophysiology of AD (Dokholyan et al., 2022). Two of the most popular hypotheses regarding the pathophysiology of AD are the amyloid cascade hypothesis and the tau hypothesis. These suggest that the presence of intracellular neurofibrillary tangles (NFTs), created by maladaptive aggregation of tau protein and extracellular amyloid plaques, lead to the accumulation of amyloid-beta peptides. These are thought to be two of the main hallmarks of AD, and therefore are assumed to play a large part in the neurodegeneration observed in AD patients (Armstrong, 2013). Consequentially, most research regarding potential treatments is based on targeting NFTs and amyloid-beta peptides (Alzheimer's Association, "Treatments and Research," 2024).

However, more recent research has shown that amyloid-beta and tau aggregation might not be the sole cause of the symptoms of AD. Researchers also do not know if these aggregations are the earliest or most significant events. This makes it difficult to identify more specific

biomarkers and come up with site-specific drug treatments (Dokholyan et al., 2022; Frozza et al., 2018; Huang, 2020). Until there is a complete understanding of AD etiology and pathophysiology, efforts towards a cure seem to be futile.

Environmental Enrichment

Since it appears that successfully identifying and understanding the integral molecular events that initiate the downstream processes in AD will not be possible for a while, some researchers have started to explore preventative measures that can be taken to reduce individuals' risk factors for developing Alzheimer's disease (Dhana et al., 2022; Leshner et al., 2017). Particular emphasis has been placed on environmental enrichment (Lima et al., 2018). Enriched environment in animal studies is defined as "having motor, sensory, cognitive, and social stimulation" (Figuracion & Lewis, 2021). More broadly, environmental enrichment "encompasses a changing environment that encourages socialization, exercise, sensory, and cognitive stimulation" (Figuracion & Lewis, 2021). Several studies involving mice have shown that environmental enrichment providing both cognitive and physical stimulation can aid in counteracting amyloid pathology and reduce cognitive deficits seen in AD (Bayne, 2018; Herring et al., 2011; Jankowsky et al., 2005). This is thought to occur due to stimulating environments increasing synaptic activity as well as the number of synapses.

While there are countless studies using rodents as a subject model to look at the effects of environmental enrichment on a variety of behaviors, research using zebrafish is relatively scarce. Since the use of zebrafish in research has skyrocketed, the gaps in the literature have become more obvious and more important to explore. While housing methods, experimental procedures, and protocols for experiments with rodents have been established and standardized (Harland &

Dalrymple-Alford, 2020), the same cannot be said for zebrafish. There is still discussion over standardized housing and care, which therefore impacts the standardization of what is deemed to be an enriched vs standard environment (Sarma et al., 2023; Stevens et al., 2021). This can lead to contradictions in findings between articles. Until there is a universal standard for specific protocols, it is difficult to determine the best and most effective way to enrich the environment of housing for zebrafish (Stevens et al., 2021). On the other hand, we do know that zebrafish are social creatures that prefer to be grouped together, and there have been indications that the incorporation of gravel and plants help to promote their welfare (DePasquale et al., 2021).

Zebrafish (*Danio rerio*)

The zebrafish, an aquatic vertebrate native to India, has been regularly used as a model organism for over thirty years (Parichy, 2015; Poon & Brand, 2013). Zebrafish were first used in research by George Streisinger, also known as the founder of modern zebrafish research. Streisinger originally took an interest in zebrafish due to their ability to breed easily, their small size, and their swift development, featuring a transparent embryo (Burgess & Burton, 2023).

By 24 hours post fertilization (hpf), zebrafish are considered embryos and have developed the majority of their essential organs. Roughly 2 days post fertilization (dpf), the embryo hatches and enters the larval stage at 3 dpf where most organs have fully developed. At 30 dpf, they enter the juvenile stage, and reach adulthood at 90 dpf (ZFIN, 2024). Once fully matured, zebrafish are roughly 3-5 cm in length. Their small size, in addition to their social nature, makes it easy to safely house many fish in a small space (ZeClinics, 2024). This in turn adds the advantage of using larger groups during testing, allowing for greater statistical power.

While zebrafish were originally chosen by Streisinger for convenience, they have remained a primary model organism due to the advantages they offer for genetic and molecular studies (Adams & Kafaligonul, 2018; Liao et al., 2012). Drug administration can be extremely simple as small molecules can be absorbed through their skin and gills (Morikane et al., 2020). Their genomes can easily be altered and manipulated, and most importantly, they possess a high neurophysiological homology to humans. This includes similarities regarding neurochemistry, endocrinology, and genetic makeup. Specifically, humans and zebrafish share roughly 70% of the same genes, which could be an explanation for the cellular similarities observed between the two species (Friedrich et al., 2013). Most importantly for neuroscience research, zebrafish and humans have some homologous brain structures, with zebrafish possessing neurons, synapses, and regions that correspond to the human counterpart (Howe et al., 2013; Newman et al., 2014).

Spatial Memory

One of the types of memory affected by Alzheimer's disease is spatial memory (Jahn, 2013), defined as the "storage and retrieval of information within the brain that is needed both to plan a route to a desired location and to remember where an object is located or where an event occurred" (Bisby & Burgess, 2024). The hippocampus is thought to play a major role in spatial memory, as it has been demonstrated that damage to this area results in difficulties with spatial orientation and navigation (Shrager et al., 2007). The cholinergic system is also an essential component in spatial memory, as it helps to modulate hippocampal function through acetylcholine (Mueller et al., 2004).

It is thought that AD impacts spatial memory because it can lead to atrophy in hippocampal regions (Silva & Martinez, 2023). There is also a well-documented atrophy of

cholinergic neurons, resulting in a decrease in acetylcholine in the hippocampus (Zhu et al., 2017). This can lead to difficulties consolidating and retrieving memory, resulting in problems with spatial orientation and navigation (Ferreira-Vieira et al., 2016; Haam & Yakel, 2018; Maurer & Williams, 2017). This type of brain damage leading to memory impairments can be replicated in animal studies through cholinergic antagonists. By administering a cholinergic antagonist, the effects seen from cholinergic atrophy can be simulated in experiments, therefore making it possible to conduct research regarding early symptomology of AD (Bajo et al., 2015; de Abreu et al., 2018).

As a result, studying spatial memory in animals has become a popular research avenue due to its potential for pharmacological and clinical applications (Lewis et al., 2017). There are many studies that use rodents to study spatial memory, resulting in the development of several different mazes (Lennartz, 2008). Some of these techniques, while ideal for rodents, are difficult to implement in research with zebrafish. Rodents are easy to transport and handle without increasing anxiety or stress and can be easily externally motivated (Sensini et al., 2020; Spangenberg & Wichman, 2018). Zebrafish, however, are easily stressed, especially regarding transport and handling, and controlling for external rewards is much more challenging in an aqueous environment (Clark et al., 2012; Horstick et al., 2018). Therefore, mazes such as the Y-maze are commonly used to study spatial memory through spontaneous alternation, which limits the amount of handling, and does not require external motivation (Cognato et al., 2012).

Continuous Spontaneous Alternation Behavior in the Y-Maze

Spontaneous alternation behavior (SAB) is "the tendency to systematically alternate directional choices in successive maze arms" (Rothacher et al., 2020). These types of assessment

offer the benefit of not needing an external reward or motivator, as there is no right or wrong choice to be made. While there are several different types of mazes used in these tasks, one that is commonly used to test zebrafish is the Y-maze (Cognato et al., 2012). The Y-maze allows for the subject to freely explore its surroundings for a given amount of time, also known as continuous spatial alternation (Behavioral and Functional Neuroscience Laboratory, 2024). Other maze configurations, such as a T-maze, can also be used. When the T-maze is used, the subject, typically a rodent, is placed in the middle of the maze and chooses an arm of the maze to visit. The subject is then picked up and this process is repeated (d'Isa et al., 2021). For subject models such as zebrafish, it is best to limit handling as much as possible to avoid anxiety-related novelty avoidance (Cleal, Fontana, Ranson et al., 2021). Pairing this consideration with the benefit of not needing an external reward makes continuous spatial alternation tasks a preferred testing method when using zebrafish as a subject model.

Spontaneous alternation tasks are particularly beneficial for spatial memory studies. The underlying theory, which has been supported by decade's worth of results from rodents and nonhuman primates, is that spatial memory is indicated through exploratory behavior. If the subject alternates between all three arm entries in a manner that is greater than chance, this indicates that the subject is remembering the most recently entered arms and chooses to explore the arm that was least recently entered (Kim et al., 2023). In other words, it is thought that subjects with proper spatial memory will exhibit alternating behavior at a level greater than chance (see Figure 1). This type of behavior is referred to as spatial alternation.
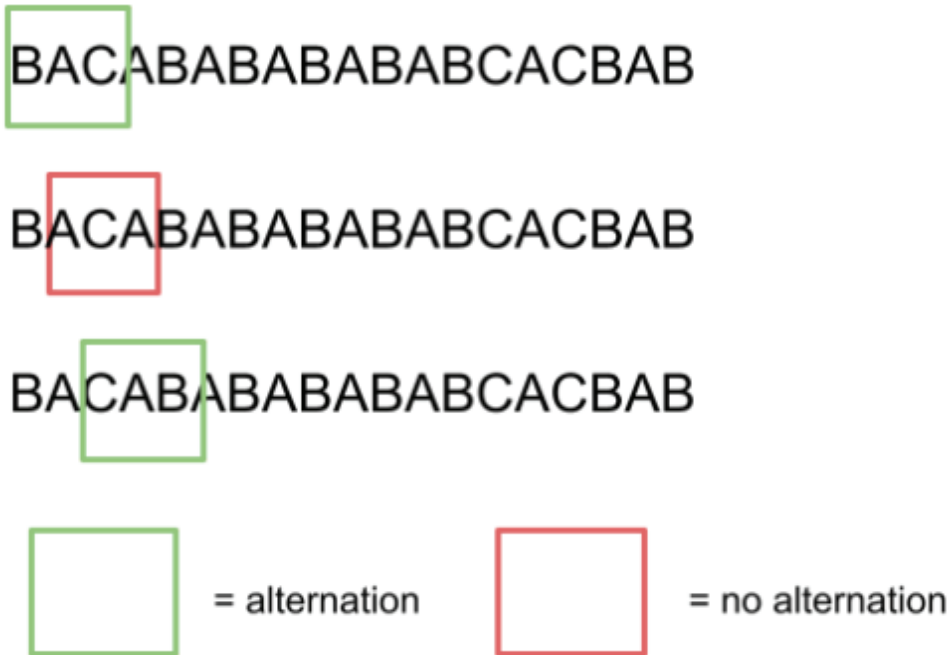
Figure 1. Spatial alternation example. The three entries within the green box demonstrate an alternation, and the three entries within the red box do not demonstrate alternation. The zebrafish must enter all three arms within three choices for their movement to be classified as an alternation. The box is a visual representation of the sliding window that is used to code test behavior. Image from Ancheril (2023).

Through these spontaneous alternation experiments, rodents, primates, birds, and *Drosophila* have all exhibited exploratory behavior through spatial alternations, therefore being a good subject choice for examining spatial memory (Cleal, Fontana, Ranson et al., 2021). It was assumed that zebrafish would also show spatial alternation behaviors, but surprisingly, there are no published reports of this behavior in zebrafish. Unpublished studies in our lab have shown inconsistent results (Ancheril, 2023; Moore, 2021). Moore (2021) did find a tendency toward spatial alternation behavior in fish, but only when multiple zebrafish were tested as a group. When tested individually however, no spatial alternation behavior has been observed or reported.

Some researchers decided to take a different approach to studying spatial memory in zebrafish, specifically the Parker Lab. This group started looking at a different type of alternation, which will be referred to as response alternation. This type of alternation is based on the sequence of right and left turns that are made instead of the specific maze arms that are entered. Therefore, the behavior of the zebrafish can be reflected through a series of turns as opposed to specific maze arm choices (Cleal et al., 2023). When looking at spatial alternation, three arm entries are analyzed at a time. Response alternation, on the other hand, has been measured as a sequence of four turns, referred to as a tetragram (see Figure 2). Using Parker's methodology, there are 16 possible tetragrams (shown in the horizontal axis of Figure 6). The specific tetragrams important for analyzing alternations are LRLR and RLRL, and for measuring response repetitions are LLLL and RRRR.

Figure 2. Tetragram. Arm entries are translated into a series of left (L) and right (R) turns. The tetragrams RLLR, LLRR, and LRRL are highlighted in the sliding window, and this method is carried through the entire sequence. Image from Ancheril (2023).

The tetragrams LRLR and RLRL (referred to as response alternations) appear to be a measure of spatial memory, whereas the tetragrams LLLL and RRRR (referred to as response repetitions) are considered a measure of response perseveration (Cleal, Fontana, & Parker, 2021). Perseveration is defined as making the same response over and over. After data analysis, if the number of all tetragram types observed do not significantly differ, then the behavior is determined to be random. If the tetragrams for response alternations occur at a significantly higher rate than the tetragrams for response repetitions, this can be an indication of spontaneous alternation behavior (Fontana et al., 2019; Fontana et al., 2021; Fontana et al., 2022).

Scopolamine

Scopolamine ($C_{17}H_{21}NO_4 \cdot HBr$) is a relatively selective and reversible antagonist of muscarinic cholinergic receptors (Tullberg, 2007). It is an FDA approved medication for treating motion sickness and postoperative nausea (Riad & Hithe, 2024). While serving as an effective antiemetic, scopolamine can also cause transient cognitive amnesia and electrophysiological changes that replicate neurophysiological changes seen in AD (Bajo et al., 2015; de Abreu et al., 2018). For the present study, the main effect of interest is the impairment of spatial memory via decreasing levels of acetylcholine activity at muscarinic receptors (Alikatte et al., 2012; Braida et al., 2014; Olson et al., 2023; Sun et al., 2021). This makes its use part of a solid behavioral model to study some of the cognitive impairments from Alzheimer's disease (Bortolotto et al., 2015; Caramillo, 2017).

Hypothesis

To summarize: (1) AD is characterized, in part, by impaired memory, and spatial memory is especially affected. (2) Scopolamine can induce spatial memory impairments in animals and there is widespread agreement that these effects can serve as a model of some aspects of AD. (3) Exposure to environmental enrichment is being investigated as a possible treatment to slow the progression of AD. Therefore, the hypothesis for this study was that cognitively impaired zebrafish (caused by administration of scopolamine) that are reared in an enriched environment, will demonstrate spontaneous alternation in a Y-maze task, whereas those reared in an impoverished environment will not.

MATERIALS AND METHODS

Subjects and Housing

Forty juvenile zebrafish (*Danio rerio*), 16 days post fertilization (dpf) at the beginning of the experiment, were used as subjects. Subjects were bred in-house using adult breeder fish obtained from a local PetSmart. These fish were housed in the lab for at least one month prior to breeding.

Adult fish were maintained in 10-gallon tanks filled with system water. System water was prepared by adding 63 grams of Instant Ocean sea salts to 30 gallons of RO/DI (reverse osmosis deionized water) to create a salinity level of 0.53-0.56 ppt. Then, 70 g/L sodium bicarbonate, 5 mL Prime, and 10 mL Fluval water conditioners were added. Water temperature and pH levels were maintained within the ranges 26-28 °C and 6.8-7.2. The housing room was on a 14:10 hour light-dark cycle with light onset at 0600h.

For breeding, adult fish were separated by sex into 5-gallon tanks where they remained for 72 hours. During this time, they were fed with TetraMin flakes supplemented with spirulina

twice per day. Once per day they were given frozen brine shrimp. In the evening of the third day, a 5-inch x 7-inch tray filled with marbles was inserted in the bottom of the females' tank. The tray contained a plastic plant. Males were then transferred to the females' tank in a ratio of 2F:1M. They were left undisturbed overnight. In the morning the marble tray was removed, and eggs were collected using a pipette. Eggs were rinsed twice in clean system water and transferred to a 6 x 22 x 16-inch (height x length x width) polycarbonate tank kept in a water bath to maintain temperature.

Embryos/larvae were not fed from 0 dpf to 5 dpf. During this time, the yolk provides their nutritional needs. Starting on 6 dpf, they were fed twice a day with baby/fry food consisting of equal parts (a) spirulina, algae, veggie baby and fry food [Aquatic Foods, Inc], (b) plankton powder [California Blackworm Company], and (c) freeze-dried rotifers [Aquatic Food, Inc]. This was alternated with pre-packaged Hikari First Bites. At 10 dpf, frozen baby brine shrimp were added to the diet. On 21 dpf, the fish were switched to juvenile food (baby food plus Golden Pearls 200-300 micron [Aquatic Foods, Inc]).

At 16 dpf, the fish were moved to four 5-gallon tanks (10 per tank). Roughly 50% of the tank water was replaced on a weekly basis. All four tanks contained a heater and bubble filter. Two of the tanks were environmentally enriched, and the other two were environmentally impoverished (see Figure 3). The two environmentally enriched tanks were set up in the following way: The floor was covered with gravel, and each tank always had at least one plastic plant. An assortment of different toys (rubber balls, tubes, pots, different types of fake plants, suction-cupped wall décor) were swapped around every 4 days (see Figure 4). This timeline was established to prevent increasing stress levels in the zebrafish by disturbing them too often,

giving them time to adjust to the new stimuli, while also not letting too much time pass where

the novelty would potentially wear off (Cleal, Fontana, Ranson et al., 2021).

Figure 3. Tank setup. The two environmentally enriched tanks are on the left, and the two environmentally impoverished tanks are on the right. The enriched tanks contain gravel and different kinds of plants and toys. The nonenriched tanks are covered to minimize visual stimuli.

Figure 4. Enriched tank. A closer view of an enriched tank during the experiment. Objects were swapped out every four days.

The two tanks that were environmentally impoverished were placed in a darker corner of the housing room, with tan paper taped around the sides to prevent the fish from seeing other fish in the surrounding tanks. The front of these two tanks were covered in green mesh. These tanks only contained the filter and heater. The only other enrichment was provided by the other fish in the tank.

This experiment entailed a two-by-two setup, with 20 zebrafish growing up in an enriched environment, and 20 growing up in an impoverished environment. 10 fish from each group were exposed to scopolamine for 30 minutes prior to testing. The remaining 10 fish from each group were exposed to system water for 30 minutes prior to testing.

Drug Administration Procedure

The dosage and exposure time of scopolamine were based on the current literature regarding scopolamine use in zebrafish (Bortolotto et al., 2015; Caramillo, 2017; Cognato et al., 2012). A 200 µM solution of scopolamine was used. This was determined based on the observations that 200 µM was high enough to impair memory function, but low enough to prevent negative effects on the motor system. Fish were exposed for 30 minutes immediately before behavioral testing in the Y-maze.

Scopolamine was diluted with water into beakers that were kept at room temperature. Immediately before exposure, 98 mL of water and 2 mL of a stock solution of 10 mM of scopolamine were mixed to achieve the desired concentration of 200 µM. Fish were removed from their home tank and placed into a 200 mL beaker containing the 100 mL solution to ensure that a fish would be unable to jump out. After the 30-minute exposure time, the fish were removed from the beaker, rinsed in normal system water to wash away any excess solution from their bodies, and immediately placed in the Y-maze. The solution in the beakers used for exposure along with the system water used for rinsing were emptied into a waste bucket for proper disposal. The same was done for the control group, where only system water was used. They were placed in a beaker for 30 minutes prior to testing, rinsed off, and then placed in the Y-maze. The water from those beakers was also emptied into the waste bucket.

Y-maze

        The Y-maze, built by me in the fall of 2021, was made from black corrugated sheets that were cut to give the maze arms dimensions of 15.5 x 23 x 9.5 cm (height x length x width) and held together with clear aquarium silicone sealant. The arms of the maze were arranged to form 120º angles, forming the shape of a Y. This was fixed to a base of white plastic, secured with the same silicone sealant, to prevent fish from escaping. The entire maze apparatus was then placed into a foldable dog pool with a diameter of 63.5 cm and height of 19 cm. The pool and the Y-maze were filled with 12 L of system water, and three flat heaters (Aqueon 7.5W) were placed outside of the maze on the plastic base to maintain the water temperature, one heater between each arm (see Figure 5).
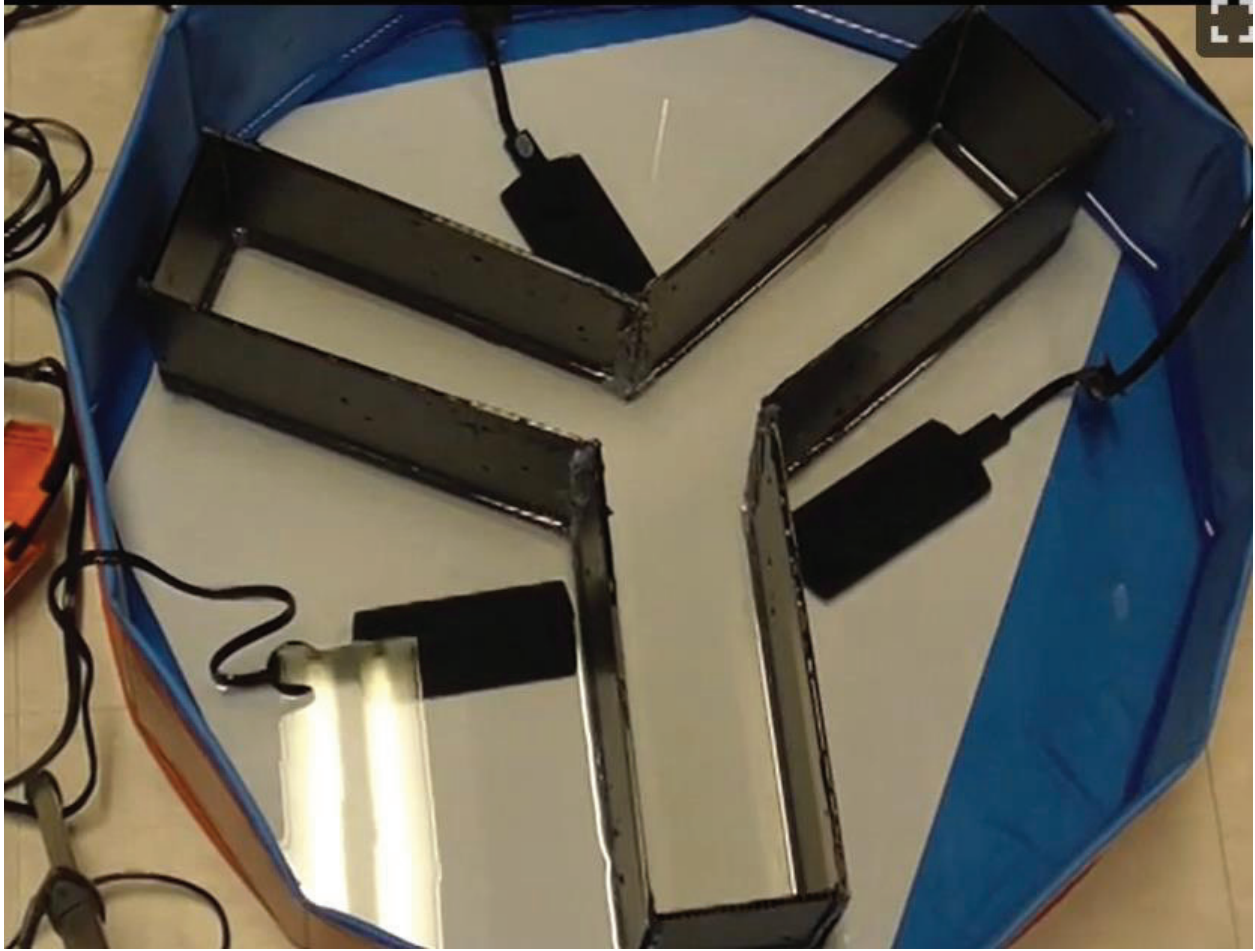
Figure 5. Experimental Setup. The Y-maze was placed in the pool, with heaters on each side.

Spontaneous Alternation Task

Fish were tested when they reached 46-50 dpf. 40 fish were originally divided among the four treatment tanks (10 each), but 3 had died prior to the start of testing. Sample sizes ranged from 8-10 per group: 10 from the enriched environment exposed to water prior to testing, 10 from the enriched environment exposed to scopolamine prior to testing, 9 from the impoverished environment exposed to water prior to testing, and 8 from the impoverished environment exposed to scopolamine prior to testing.

After the 30-minute drug exposure time, an individual zebrafish was placed in the Y-maze for 8 minutes, and movements were recorded by a video camera placed on a tripod above the maze. After testing, video records were scored by recording the sequence of maze arms that were entered by the fish during the 8-minute test. The arms were designated as A, B, and C. An arm entry was defined as the fish completely entering into the arm. Repeat entries (going in to the middle and reentering the same arm) were not counted.

After recording the sequence of entries, the percent of spatial alternations was calculated. This was done by taking the number of alternations and dividing it by the number of possible alternations (total number of arm entries - 2) x 100.

In order to look at response alternation, the sequences of arm entries were rewritten into sequences of left (L) and right (R) turns (see Table 1). The tetragrams from these sequences were recorded via a sliding window of 4 choices. The frequency of occurrence of each tetragram type was recorded for each subject.

Movement	A → B	A → C	B → A	B → C	C → A	C → B
Turn Direction	R	L	L	R	R	L

Table 1. Arm entries were transcribed into left (L) and right (R) turns using this method. Image from Ancheril (2023).

RESULTS

Arm Entries

Recordings of the Y maze tests were viewed, and the sessions were scored for arm entries. The three arms of the maze were designated as "A," "B," and "C" and the order of entries was recorded for the entirety of the 8-minute test. The total number of arm entries was analyzed using a 2 (enrichment condition) x 2 (pre-test drug) between-groups Analysis of Variance (ANOVA). The analysis yielded no significant effects [largest $F (1, 30) = 1.28$, $p = .27$]. All fish, regardless of enrichment experience or drug showed the same number of arm entries ($M = 33.4$, $SE = 2.1$).

Spatial Alternation

The number of 3-choice spatial alternations recorded for each subject was converted into a percent as defined by Lennartz (2008):

$$\% \text{ alternations} = [\# \text{ alternations} / (\# \text{ arm entries} - 2)] \times 100.$$

The 2 x 2 ANOVA indicated no significant effects [largest $F (1, 30) = 1.31$, $p = .26$]. Fish showed, on average, 53% alternations ($SE = 2.3$) which does not differ from chance performance that is defined as 50%.

Response Alternations and Repetitions

The order of arm entries for each fish was converted into a series of left (L) and right (R) turns. As described by Parker and colleagues (Fontana et al., 2019; Fontana et al., 2021; Fontana et al., 2022), the data were converted into tetragrams (a series of 4 turns). There are 16 possible patterns of 4-arm choices, as shown in Figure 6. The two types of tetragrams that are of particular interest are called response alternations (RLRL and LRLR) and response repetitions (RRRR and LLLL). Previous studies in our lab have replicated Parker's findings that adult

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zebrafish show a greater number of response alternations and repetitions, compared to all other types of tetragrams (Moore, 2021).
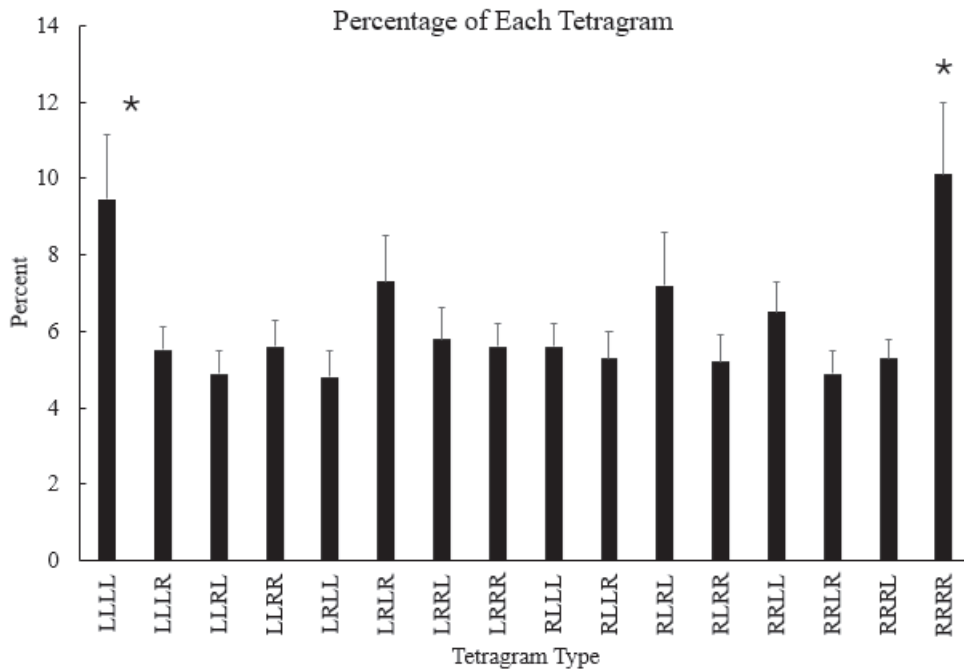

Figure 6. The percentage of each tetragram. Response alternations LRLR and RLRL are slightly higher than the others, but not enough to be statistically significant. Response repetitions RRRR and LLLL are significantly more prevalent compared to the other tetragrams.

Data from this study were analyzed using a 2 (enrichment experience) x 2 (pre-test drug) x 16 (tetragram type) mixed-factor ANOVA. Tetragram type was the repeated measure. The analysis showed no effects or interactions involving Enrichment experience or Pre-test drug [largest $F (1, 30) = 1.04$, $p = .31$). There was, however, a main effect of Tetragram Type, $F (15, 450) = 2.27$, $p = .004$. The percentage of each type of tetragram exhibited by the fish, collapsed across Enrichment experience and Pre-test drug, are shown in Figure 6. As can be seen, there

was a slightly greater percentage of response repetitions (LLLL and RRRR) than other types. Figure 7 more clearly shows the average percentage of repetitions and alternations, compared to the other types of tetragrams.
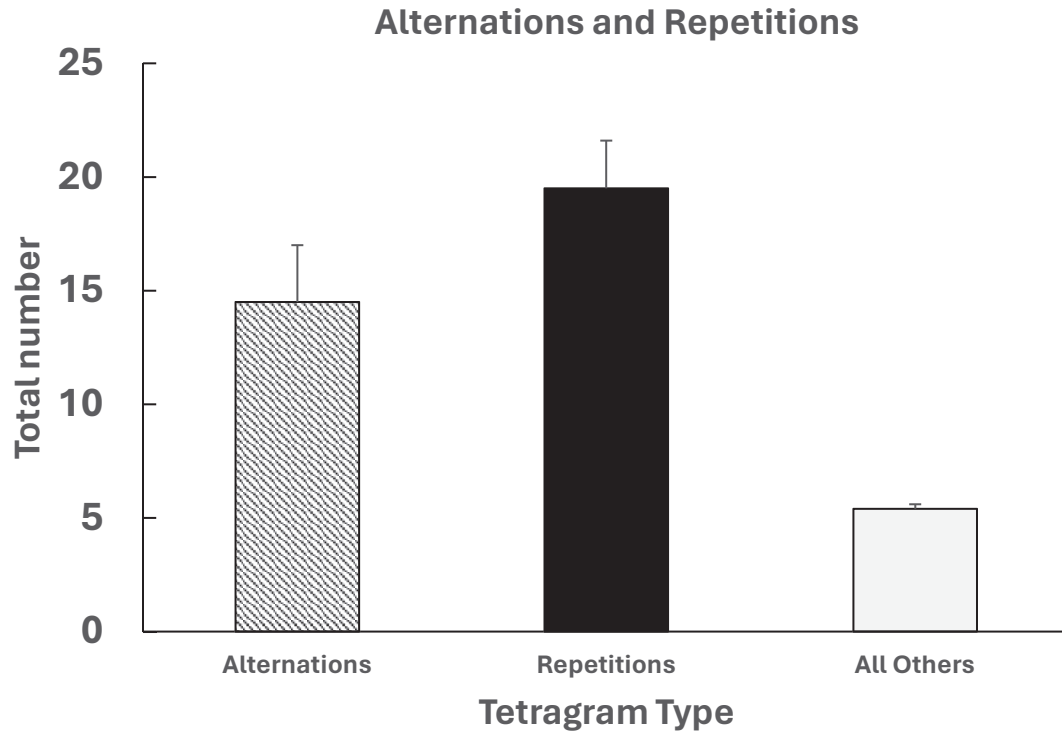

Figure 7. Average percentage of tetragrams. A slightly greater percentage of response repetitions (LLLL and RRRR) can be seen compared to response alternations (RLRL and LRLR) and the remaining 12 tetragrams (all others).

DISCUSSION

The results of this study do not support the hypothesis that cognitively impaired zebrafish that are reared in an enriched environment would demonstrate spatial and response alternation in a Y-maze task.

The results instead show that environment and drug exposure had no significant effect on performance during the behavioral task. There were no significant differences in arm entries, and the percentage of alternations bordered around chance performance (50%). This shows that zebrafish did not display spatial alternation. These findings are consistent with other studies from our lab (Ancheril, 2023; Moore, 2021), suggesting that either zebrafish do not exhibit spatial alternation, or that the methods used to test spatial alternation in other subjects (rodents, *Drosophila*, birds, humans) are not adaptable or appropriate for zebrafish.

The zebrafish also did not show response alternation based on the results from the analysis of the sequence of four sequential turns, referred to as tetragrams. The RLRL and LRLR tetragrams show a response alternation exploratory behavior. The failure to observe a greater percentage of response alternations in the fish again indicates that spatial memory was not observed. Environment and drug exposure had no significant effects on this behavior either. The lack of response alternation observed is contrary to the results observed from the Parker Lab, but this inconsistency might be due to several factors, which are discussed in the subsequent section.

The one statistically significant finding was that there were more LLLL and RRRR tetragrams (response repetitions) observed in the subjects compared to the other types of tetragrams, but there was no difference across the treatment groups. This suggests that exploratory behavior of zebrafish in the maze was based on a more perseverative response pattern than alternation. As mentioned previously, perseveration is defined as doing the same response over and over. This is a more rudimentary type of movement through the maze, one that is more indicative of a response habit than spatially-based exploratory behavior.

Limitations

While the lack of spatial alternation observed is consistent with previous findings from our lab, the lack of response alternation was unexpected. However, there are several variables that could have contributed to these inconsistencies in findings. In the present experiment, the zebrafish were between 46 and 50 dpf. Zebrafish reach adulthood at 90 dpf, so the fish that were tested here were considerably smaller than the adult fish tested previously in our lab and Parker's lab. While the zebrafish have developed all major central nervous system regions by 3 dpf, the central nervous system is still considered immature until adulthood is reached on 90 dpf. As a result, there may be some developmental changes between the juvenile and adult stage that exist, although the specifics of these changes are still being explored (Shepherd & Eisen, 2011). If this is the case, maybe zebrafish are not capable of spatial alternation before adulthood, but there have not been any findings to support this.

The Y-maze that was used in the present study was the same one that was used for testing much older (and larger) adult zebrafish in previous studies in our lab (Ancheril, 2023; Moore, 2021). That the fish in the juvenile stage are slower and smaller than fish in the adult stage may explain why there were many fewer arm entries observed. In the Moore (2021) and Ancheril (2023) studies, adult zebrafish made approximately 80 arm entries during a 10-minute test, nearly three times as many as were evident in the juvenile subjects in the present study. The fewer numbers of arm entries, and therefore fewer possible alternations and repetitions, could have influenced the findings. In the Parker Lab, the Y-maze used is very small, with dimensions of 14 x 5 x 2 cm (height x length x width) for adult zebrafish over 90 dpf, and dimensions of 0.5 x 2.5 x 1 cm (height x length x width) for juvenile zebrafish between 30 and 90 dpf. They also typically test the fish over the course of an hour, rather than the 8-10 minutes used in our lab. This results in thousands of arm entries, leading to more reliable data. If the fish in the present

study were tested in a smaller maze for a longer period of time, or tested as full-grown adults, there may have been more arm entries, which may have shown something different than the current data.

Conclusion

The results from this study failed to indicate that rearing in an enriched environment or pre-test exposure to scopolamine influenced spatial memory. There was no observed spatial or response alternation, and the response repetitions that were seen do not indicate utilization of higher cognitive processes. While the hypothesis was not supported, the use of zebrafish in research is still relatively new. If new methods or protocols are established in the future that succeed in demonstrating spatial alternation, this study may show different results if repeated in the future.

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