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Article

A Crossover Study Comparing the Effect of a Processed vs. Unprocessed Diet on the Spatial Learning Ability of Zebrafish

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SUMMARY

The current study aimed to compare the short-term effects of processed versus unprocessed food on spatial learning and survival in zebrafish (Danio rerio). Given public concern regarding processed foods, the results of the current study can inform consumer decision-making. We hypothesized that an unprocessed diet would improve learning and survival. Zebrafish were randomly assigned to a diet of brine shrimp flakes (processed) or live brine shrimp (unprocessed). Spatial learning was evaluated throughout the study by recording fish decisions (correct vs. incorrect) and time taken for decision. Our results show no statistically significant difference in the proportion of correct decisions or in the time taken to make decisions when the groups are compared. Notably, fish receiving unprocessed food had significantly lower mortality than those receiving processed food (p = 0.027). We concluded that while the zebrafish make progressively better and faster decisions, diet type does not contribute to improved learning. However, consumption of unprocessed diet may have survival benefits in stressful environments. Future studies may further analyze this association, utilizing larger sample size and longer study duration to clearly assess the effects of long-term exposure to an unprocessed vs. raw unprocessed diet on learning. While it is an association that needs further evaluation, the current study indicates the potential benefits of an unprocessed diet in coping with stress. Considering that zebrafish are effective models of human cognition, this has implications for human consumption of processed vs. unprocessed foods as well.

INTRODUCTION

The scientific community has extensively studied the impacts of processed foods on human health (1). Studies have found that the consumption of processed foods could potentially have an adverse effect on the human brain by lowering cognitive abilities such as memory, executive function, and intelligence quotient (2, 3). Past analyses have also indicated that, among mice, fatty and processed foods can increase the risk of dementia by preventing neurons from responding to the hormone insulin (4).

Food processing transforms raw foods and materials into more easily consumable products (5). Specifically, food processing exposes foods to extreme heat, light, or oxygen. Due to this process, the nutritional value of processed foods is significantly reduced compared to raw, unprocessed foods (5). Nutrients such as omega-3 fatty acids are an important component of all human cell membranes (6). Compared to unprocessed foods, processed foods tend to have significantly higher omega-6 to omega-3 fatty acid ratios, meaning they have proportionally lower amounts of the healthier omega-3 fatty acids (7). Omega-3 fatty acids have also been shown to improve learning and memory and mitigate the risk of mental disorders, such as depression (8).

Zebrafish (*Danio rerio*) are an ideal animal model with which to study cognition, given their physiological similarities to humans (9). Specifically, the functions of the amygdala and habenula in informing affective behavior in zebrafish are similar in humans (10). The amygdala and habenula both are components of the reward brain circuit, something that was directly assessed in our study with the punishing vs. rewarding arms of the bifurcated T-maze (11). Zebrafish exhibit similar cognitive tendencies to those of mice, which are the traditionally utilized model for human cognition (12). For instance, in the conditional place preference test, zebrafish, like mice, demonstrate preference for a location associated with a certain substance or reward (13). However, zebrafish are a preferable animal model given their cost-effectiveness (14). Other studies using zebrafish to assess cognition have utilized a bifurcated T-maze with a punitive and rewarding arm, or similar techniques (15,16,17). The bifurcated T-maze is a validated tool to assess the spatial learning ability of zebrafish, as it assesses the ability of the fish to recall color-based association of a certain location with a reward (15,18,19). The maze structure is simple—the fish is initially positioned in the longest arm of the T-maze and has two path options. The fish is punished if it takes one path, or rewarded if it takes the other. Earlier studies have indicated that a high-fat diet can contribute to reduced cognition among zebrafish (18, 20,21). Specifically, fish receiving a comparatively higher-fat diet exhibited significantly worse performance in the active avoidance test (an indicator of short-term learning and memory) (18). However, there is limited data on the effects of processed foods on the cognitive abilities of zebrafish.

Our study assessed the effects of processed versus unprocessed food diet on the spatial learning ability and

Table 1. Inter-tank analysis of the proportion of subjects who chose the rewarding arm and time taken to choose. CI: Confidence Interval; SD: Standard Deviation. Time values are reported as the mean±SD.

		Unprocessed Diet	Processed Diet	Odds Ratio (95% CI)	p-value
Phase I Learning Assessment	Proportion of Rewarding Decisions (%)	36.73	34.04	1.13 (0.49-1.4)	0.78
	Time Taken to Make the Rewarding Decision (sec.)	42.4±21.6	58.9±49.2		0.23
Phase II Learning Assessment	Proportion of Rewarding Decisions (%)	60.61	75.61	0.5 (0.18-1.35)	0.17
	Time Taken to Make the Rewarding Decision (sec.)	36.3±40.6	20.1±19		0.11

mortality of zebrafish. To assess learning, we recorded whether fish chose the correct (right) arm of the bifurcated T-maze and the time taken to make this decision for each learning assessment in the maze. In addition to assessing learning, we also observed a statistically significant difference in mortality among fish receiving processed versus unprocessed diets. We utilized a crossover design to robustly assess the effect of diet on spatial learning, distinct from past studies. In other words, the two groups of fish were exposed to both processed and unprocessed diets. We hypothesized that the group of zebrafish that were fed an unprocessed diet would exhibit superior learning abilities and better survival. Ultimately, we found that most fish, regardless of diet, tended to learn to make the correct decisions in the maze by the end of the study. Yet, fish receiving the unprocessed, unprocessed diet exhibited a lower mortality rate, indicating

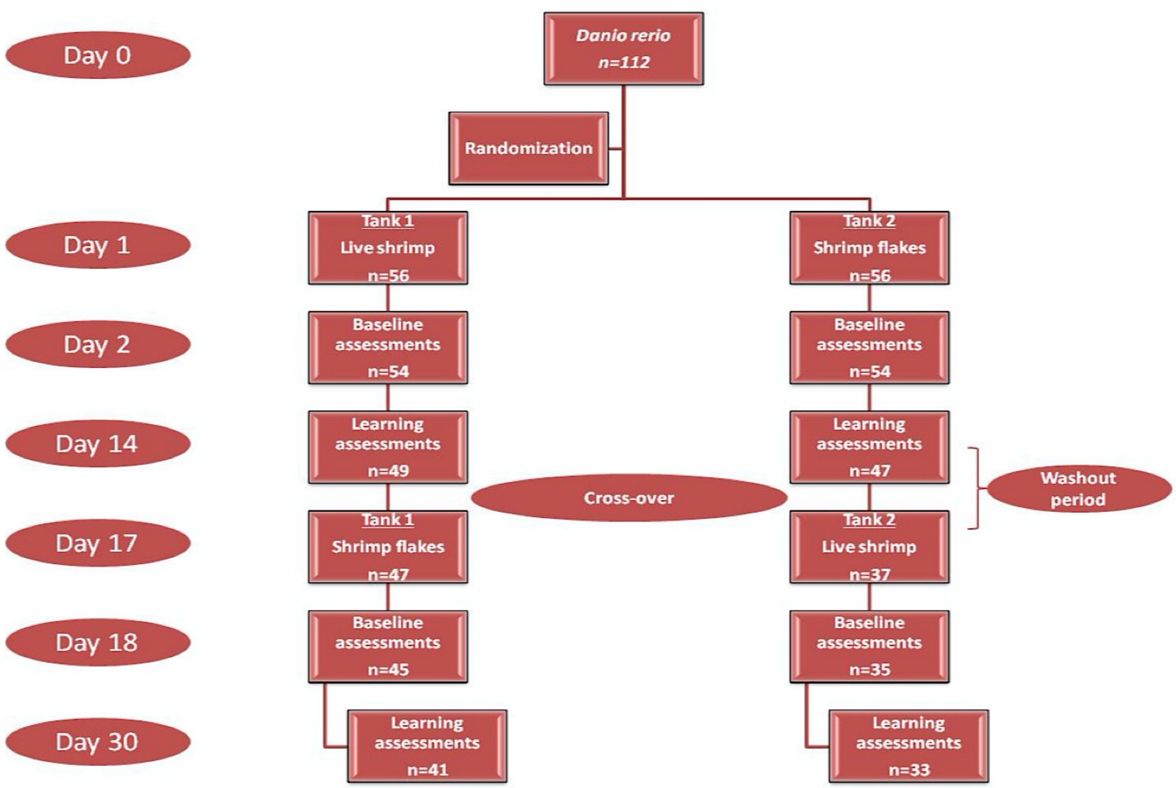

Figure 1. Flow chart depicting the study procedures, timeline, treatment arms, and subjects during each phase. The study began with 112 subjects randomized to Tanks One and Two. Both tanks received a both processed flake food and live shrimp diet. In Phase I, Tank One received live brine shrimp while Tank Two received processed shrimp flakes. After completing learning assessments and a washout period, Phase II of the study began. In Phase II, the fish switched diets and the same assessment process was repeated.

Table 2. Intra-tank analysis (Phase I vs. Phase II) of proportion of subjects who chose the rewarding arm and time taken to choose. Tank One received an unprocessed diet in Phase I and a processed diet in Phase II. Tank Two received a processed diet in Phase I and an unprocessed diet in Phase II. CI: Confidence Interval; SD: Standard Deviation. Time values are reported as the mean±SD.

	Time Taken to Make a Rewarding Decision (sec.)			Proportion of Rewarding Decisions (%)			
	Phase I Learning Assessment	Phase II Learning Assessment	p-value	Phase I Learning Assessment	Phase II Learning Assessment	Odds Ratio (95% CI)	p-value
Tank One	42.4±21.6	20.1±19	0.0009	36.73	75.61	75.61	0.0002
Tank Two	58.9±49.2	36.3±40.6	0.15	34.04	60.61	60.61	0.02

potential health benefits of unprocessed foods in coping with stressful environments among zebrafish.

RESULTS

In this randomized blinded study with a crossover design, two groups of zebrafish experienced interventions and assessments to determine their cognitive ability while consuming different types of food supplements. Tank One received an unprocessed diet of live brine shrimp for Phase I of the study (**Figure 2A**). Tank Two received a processed brine shrimp diet. The two tanks switched diets in Phase II of the study (**Figure 1**). In Phase I of the study, we colored the right arm of the T-maze connecting to the fishbowl green and the left arm red (**Figure 2B**). In Phase II of the study, we colored the right arm red, and the left arm green (**Figure 2C**). In both phases, if the fish chose the right-side arm, it was rewarded by being allowed to remain in the comfortable fishbowl environment. If the fish chose the left-side arm, the behavior was discouraged by placing a rod in the maze and vigorously swirling the water surrounding the fish. The change in the color of the T-maze arms allowed the second baseline assessment and intervention to be unique from the first baseline assessment and mitigate the effect of learning acquired during Phase I for both study groups.

Among 112 study subjects, 84 completed Phase I (75%), while 74 fish completed both phases of the study (66.1%, **Figure 1**). The remaining 38 fish (33.9%) did not survive until the end of the study. Overall trends for the study cohort

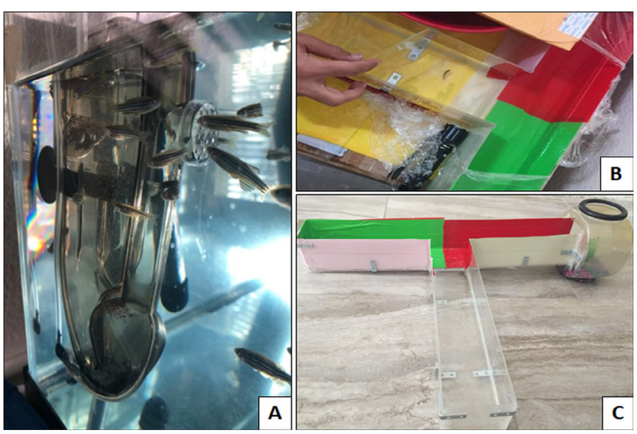

Figure 2. Study components, including bifurcated T-Maze and live brine shrimp hatchery. (A) Image of the live brine shrimp hatchery and feeder that were used for feeding an unprocessed diet to the fish during the study. (B) T-maze used during the Phase I learning assessments (right arm is green); one of the study subjects is seen swimming up the long arm of the T-maze. (C) The T-maze during the Phase II assessments (right arm is red) with one of the short arms attached to a bowl (location of the rewarding experience).

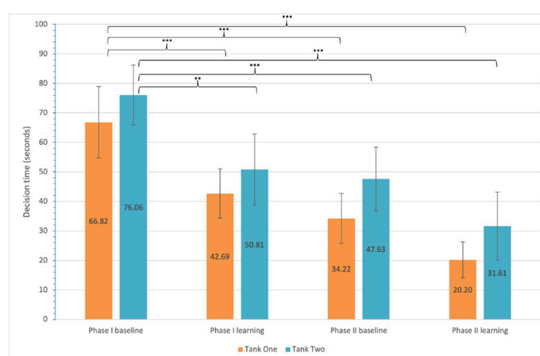

Figure 3. Bar chart showing the mean time taken (seconds) by the subjects to choose either arm during the various assessments in the study. All t-test comparisons with the first assessment were statistically significant (*** $p < 0.001$, ** $p < 0.01$). In Phase I of the study, Tank One received an unprocessed diet and Tank Two received a processed diet. In Phase II, Tank One received a processed diet while Tank Two received an unprocessed diet.

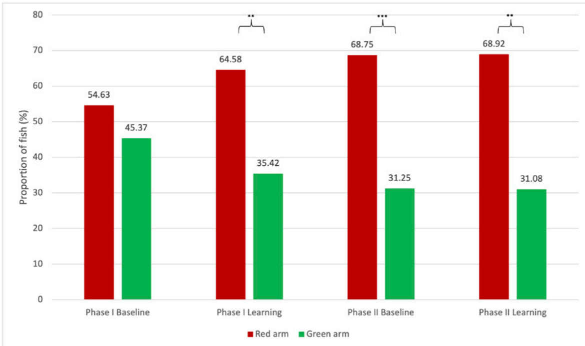

Figure 4. Bar chart depicting the proportion of fish in both tanks that choose the red or green arm during T-maze assessments. The proportions for each assessment were calculated by combining data from both tanks. Statistically significant results of the chi-square goodness of fit test are indicated (*** p < 0.001, ** p < 0.01). A statistically significant deviance from expected proportions of fish choosing each arm was not observed in the Phase I baseline assessment.

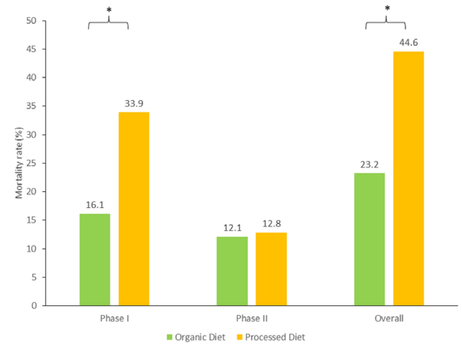

Figure 5. Bar chart showing mortality rate for fish in both tanks across Phases I and II. T-test comparisons of mortality rate between fish receiving an unprocessed and processed diet in each phase (* p < 0.05). Statistical significance was not observed in the t-test comparison for Phase II.

indicated that irrespective of the diet, the subjects made progressively faster decisions with a reduction in the time to choose either of the arms in both groups (**Figure 3**). For each tank individually, we made three T-test comparisons of time taken to make a decision in the maze: Phase I baseline vs. Phase I learning assessment, Phase I baseline vs. Phase II baseline, and Phase I baseline vs. Phase II learning assessment. For both tanks, all T-test comparisons with the Phase I baseline assessment achieved statistical significance (p < 0.01) (**Figure 3**). Regardless of diet, the fish got progressively faster in making decisions in the maze.

Inter-Tank Analysis

Overall, the type of diet did not have a statistically significant association with the proportion of subjects choosing the rewarding arm during learning assessments in Phase I (Chi-Square test, p = 0.78) or in Phase II (Chi-Square test, p = 0.17, **Table 1**). Similarly, the time taken to choose an arm did not differ between the subjects on unprocessed food versus processed food in Phase I (t-test, p = 0.23, Table 1) or in Phase II (t-test, p = 0.11, **Table 1**).

During the intra-group comparisons, the proportion of subjects that chose the rewarding arm and the time taken to make the choice exhibited statistically significant differences between Phases I and II.

In comparing the learning assessments for Tank One between Phase I (unprocessed diet) and II (processed diet), the time taken to choose the rewarding arm decreased significantly between the two phases (t-test, p = 0.0009, **Table 2**). In addition, the proportion of subjects choosing the rewarding arm also increased significantly between the two phases (Chi-Square test, p = 0.0002, **Table 2**). Similarly, the proportion of subjects choosing the rewarding arm in Tank Two also significantly increased between Phase I (processed diet) and II (unprocessed diet) (Chi-Square test, p = 0.02, **Table 2**). Finally, the time taken to choose the rewarding arm was again lower during Phase II, but it did not achieve statistical significance (t-test, p = 0.15, **Table 2**). One interesting result was that irrespective of whether it was the punitive or rewarding arm, a higher proportion of fish, regardless of diet, consistently chose the red-colored arm over the course of the study (**Figure 4**). Upon conducting a chi-square goodness of fit test, we found that the proportion of fish choosing the red and green colored arms appears to deviate from expected proportions in the Phase I learning (p = 0.004), Phase II baseline (p = 0.0007), and Phase II learning assessments (p = 0.001) (**Figure 4**).

Survival Rate Analysis

We noticed an association between the consumption of unprocessed food, irrespective of the tank environment, and overall lower risk of mortality during the study. There was a 23.2% mortality rate among fish receiving an unprocessed diet compared with 44.6% among those receiving the processed diet (OR: 95% CI: 2.7, 1.2-6.0; p = 0.027, **Figure 5**). There was not an observed statistically significant difference in mortality in Phase II. Hence, the primary cause of this overall observed difference in the study was outcomes during Phase I. In Tank One, mortality was 16.1% for fish in Tank One receiving the unprocessed diet versus 33.9% for the fish in Tank Two receiving the processed diet (OR: 95% CI: 2.7, 1.1-6.6; p = 0.048, Figure 5). However, mortality rates were comparable among the groups during Phase II. We observed a 12.1% mortality rate for fish in Tank One receiving the processed diet and 12.8% for Tank Two receiving the unprocessed diet (OR: 95%; CI: 1.2, 0.3-4.6; p = 1, **Figure 5**).

DISCUSSION

Within our study, the diet of the zebrafish did not affect the number of correct decisions or the time it took to make these decisions. We also saw that none of the inter-tank comparisons in either phase showed a statistically significant difference in the proportion of correct decisions or the decision time between the two groups fed different types of food. Furthermore, irrespective of the diet, we observed a statistically significant increase in the proportion of subjects that chose the rewarding arm and a statistically significant decrease in the time taken to make that choice. Overall, the subjects became faster and improved in making decisions with subsequent assessments irrespective of the type of food in their diet. This effect could indicate an adaptive response or a learned reflex (22). Baker et al. found that differences in how zebrafish cope with stress (reactive vs. proactive coping styles) can influence learning abilities and memory (16). The increase in quickness with which fish made a decision may be an adaptive response to the stresses of the T-maze.

While there is little in the literature regarding the effect of processed versus unprocessed food on zebrafish learning ability, past studies found associations between consumption of unprocessed food and improved cognitive development among children (23, 24). There could be several reasons for the lack of impact of diet on learning abilities in our study, the most pertinent of which appear to be the sample size and the duration of the study. Due to funding and time constraints, we designed the study as a pilot which likely left the study under-powered to show a statistically significant difference. The fish had limited time to adjust to the conditions of the tank, which may have contributed to the high mortality rate which reduced our ability to demonstrate statistical significance.

Furthermore, we were forced to limit the data collection period to 30 days, which led to relatively short durations of the interventions (i.e., time period over which the fish received each diet type)during each phase. This may have limited the ability of diet to impact the learning abilities among the subjects. A similar study published by *Meguro et al.* assessed the effect of a high-fat diet on zebrafish learning. The subjects were fed for a significantly longer period of 11 weeks (18). They observed significant impairment of cognitive function, as measured by the active avoidance test. It is possible that cognitive effects arising due to diet take longer than the duration used in the current study to demonstrate a difference. There is specific reasoning behind using such long feeding periods in this category of research. Industrial processing of foods can cause the removal of specific nutrients crucial to brain function and overall health. It is reasonable to conclude that fish need to be deprived of such nutrients for a longer period of time before their effects can be observed. *Louzada et al.* identified a significant decrease in consumption of valuable micronutrients as consumption of ultra-processed foods increased among a sample of the Brazilian population (25). These micronutrients included vitamin B12, vitamin D, vitamin E, niacin, pyridoxine, copper, iron, phosphorus, magnesium, selenium, and zinc. Low micronutrient consumption can have deleterious implications for cognition in adults. Iron, for example, is a necessary cofactor for crucial enzymes that are involved in the synthesis of neurotransmitters (26). Magnesium deficiency may affect enzymes involved in neuromuscular activity, such as ATPase enzymes (27). *Denniss et al.* found in their double-blind study that healthy adults receiving a multivitamin/vitamin D intervention exhibited significant improvement of cognitive tasks assessing memory, visuomotor processing speed, and motor planning (28). The deficiency of these crucial vitamins necessary for normal brain function takes time to occur and show its impacts on brain function and therefore may not be visible in a study with short duration.

Intriguingly, we found an association between consumption of an unprocessed diet and overall lower mortality among the zebrafish. While this difference was statistically significant for the overall duration of the study, it is noteworthy that most of the deaths occurred during the first phase of the study. This may have been driven by the increased stress of a new environment, study interventions, and subsequent evaluation. Our work has shown that an unprocessed diet may be superior to a processed diet by helping the subjects cope with stressful situations.

Furthermore, the subjects on the unprocessed diet subjectively appeared to be more active and healthier in general, although we did not have objective assessments to demonstrate this. These fish moved around the tank with greater energy and speed (fish on the processed diet appeared more lethargic) and were also more active during assessments in the T-maze. The exteriors of these fish also appeared relatively brighter and more colorful. Future studies can further assess this through Body Condition Scoring, a technique which considers the Body Mass Index of the fish (29). Locomotor activity of the fish can also be measured by tracking swimming behavior through metrics such as distance traveled, speed, and total time mobile versus immobile (30). It is pertinent that the difference in survival did not seem to be driven by the tank environment, as the mortality rate among both tanks was comparable in Phase II of the study, excluding tank environment as a potential confounding factor with diet. Serial assessments of water quality were conducted in both tanks in order to ensure that the tank environments were comparable throughout the duration of the study. We predicted that the fish being fed an unprocessed diet would appear more active and healthier overall. Yet, we did not anticipate such a high difference in mortality, leading us to hypothesize that an unprocessed diet can confer a survival advantage in terms of coping with stressful situations. It is well known that stress is a pro-inflammatory state and processed foods can increase inflammation while an unprocessed diet may have anti-inflammatory effects (31-34). This perhaps allowed the subjects consuming an unprocessed diet to survive the stress of the new environment. In an effort to parse out the putative benefits of unprocessed food in coping with stress situations, future studies may look at incorporating a 'pre-experimentation washout period' where all the fish may be treated with unprocessed food at the beginning of the study for a few days before randomizing the fish into different study arms.

Another intriguing result observed in this study was the higher proportion of fish, irrespective of diet, selecting the red arm. The trend started in the baseline assessments during Phase I itself with a progressive increase in the proportion of subjects favoring the red arm in each subsequent assessment. These trends were consistent enough to consider the possibility of zebrafish having an innate preference for red over other colors. While some studies indeed found that fish may have an aversion to certain colors, these findings are not consistent across different studies in the literature (35, 36). Future studies may consider utilizing alternate visual stimuli like vertical and horizontal lines when conducting cognitive assessments.

The current study included both a punishing and rewarding arm within the T-maze environment. Such a design does not permit conclusions regarding the basis of a decision, which limits our ability to discern differential effects of the diet. In other words, it is not clear whether the fish learned to select the rewarding arm out of a desire to avoid the aversive arm or out of a desire to experience the comfortable environment of the rewarding arm. Due to time and funding constraints, we opted for this model as opposed to first assessing the zebrafish response to the positive stimulus and then the negative stimulus. Future studies assessing zebrafish diet with a T-maze may consist of one rewarding arm and another arm which offers neither punishment nor reward to the fish. Alternatively, the maze could consist of one arm inflicting a punishment and another offering neither punishment nor reward. Future studies structured in this manner could discern if certain regions of the brain controlling desire for pleasure are affected differentially by diet compared to regions controlling a desire to avoid punishment. Specifically, the mesolimbic dopamine system mediates pleasure and rewarding experiences, while the amygdala controls emotions for fear and anxiety, and either of these systems may be more sensitive to dietary changes (37, 38).

Finally, there may be lingering effects of certain diets that may persist beyond the period that the fish were fed a particular diet. It is indeed possible that the presence of a crossover period in the current study may have led to an interaction of the effects of second diet with that of the initial diet received in the previous phase, thereby negating any potential differences. Therefore, it may be advisable to employ a non-crossover design in future studies. Notably, both groups of fish also received processed flake food during the 'washout' period prior to crossing over to the other diet—this processed diet may have influenced the learning observed during T-maze assessments in Phase II.

Future studies can build on our results by randomizing larger groups of fish on different diets for longer periods of time to more clearly assess how long-term exposure to certain foods may influence learning ability. Future studies should also further assess the potential benefits of an unprocessed diet in coping with stressful situations in zebrafish. Ultimately, although we did not observe a statistically significant difference in learning capability between the two zebrafish groups, our study design offers a novel approach to study the effect of diet on zebrafish through spatial learning assessments.

MATERIALS AND METHODS

The Scientific Review Committee associated with the Dallas Regional Science and Engineering Fair granted formal approval for the use of vertebrate animals in this study. The crossover study took place over a 30-day period and consisted of two phases (**Figure 1**).

Study Population

All zebrafish (n=112) were obtained from a local pet supplier (Pet Supplies Plus) and were received in the same shipment the day before study initiation. All fish were in good health when experimentation began. The fish were randomly assigned to Tank One (n=56) or Tank Two (n=56). The tanks were similar in size and shape, each with a 20-gallon capacity. We placed the tanks in a room with a 13-hour light/11-hour dark cycle and a room temperature of 23 °C. A Tetra® filter was attached to both tanks. A brine shrimp hatchery/feeder was attached to the side of Tank One (**Figure 2A**).

Study Procedures

The cognitive assessments were performed using a bifurcated T-maze. The maze utilized in this study was developed as per standard guidelines (dimensions: long arm 45.72 cm, short arms 30.48 cm, width of all arms 10.16 cm) using plexiglass sheets. After allowing acclimatization for 48 hours in the new tanks for both groups, Phase I of the study began. We conducted a baseline assessment and intervention using the bifurcated T-maze (**Figure 2B, 2C**) in a blinded fashion with the help of an assistant. All fish from Tank One were transferred into the maze for one hour to allow the fish to become acclimated to the T-maze environment and reduce stress during the assessments. Next, all fish were removed from the maze and kept in an intermediate holding tank. Subsequently, each fish was individually placed into the long arm of the maze. If, after 30 seconds, the fish did not leave the long arm and select the red or green arm, the fish was stimulated to move by gently tapping on the end of the long arm twice. Once the fish chose one of the two shorter arms, the time taken to do so was recorded. A 10.16 x 10.16 cm plexiglass sheet was placed behind the fish to prevent it from leaving the chosen arm. If the fish chose the green, right-side arm, we rewarded the fish by allowing it to remain in the comfortable fishbowl environment for several minutes before returning to the tank (rewarding arm). If the fish chose the red, left-side arm, the behavior was discouraged by swirling the water surrounding the fish for 10 seconds while ensuring not to hit the fish itself (punitive arm). We then placed the fish back into its home tank. This protocol was followed for all fish individually in both tanks on Day 2 of the study.

For the next 12 days, the fish in Tank One received a live brine shrimp diet (purchased from Amazon) while fish in Tank

Two received a diet of processed brine shrimp flakes (Cobalt Aquatics). Then, we conducted a learning assessment using the T-maze. The learning assessment consisted of the same procedure as the baseline assessment on Day 2 of the study, but we recorded the chosen arm and the time taken to make a decision. Following this, both tanks were fed simple tropical flake food consisting of a combination of processed nutrients (Tetra®) for three days. This served as a washout period before both groups crossed over to the other diet. After three days, Phase II of the study began. We conducted a second baseline assessment and intervention; however, the colors of the left and right arms of the maze were switched (the left-side arm of the maze was colored with green duct tape, and the right-side arm of the maze was colored with red duct tape). The change in the color of the T-maze arms was meant to mitigate the effect of learning acquired during Phase I for both study groups. Though the colors were switched, the left arm of the maze remained the punishing arm, and the right arm remained the rewarding arm. After the second baseline assessment, the fish in Tank One received processed brine shrimp flake food, and those in Tank Two received live brine shrimp for 12 days. Then, a final learning assessment was conducted using the same protocol as previous assessments.

Water Maintenance

We conducted water quality testing every two weeks for the duration of the study per the manufacturer's specifications. We utilized a water quality testing kit (API) to assess the ammonia, nitrite, nitrate, and pH levels. If these levels were found to be unacceptable (i.e., ammonia and nitrite levels outside the range of 0-0.5 ppm, nitrate levels less than 40 ppm, or pH outside the range of 7.0-7.8), then we conducted a water change of approximately 5 liters to maintain water quality and prevent any adverse effects on the fish.

Statistical Analysis

We compared the proportion of fish that chose the rewarding arm versus the punitive arm on the T-maze using a Chi-Square test. We assessed the time taken to choose an arm of the T-maze based upon fish diet using the independent t-test. In addition, the proportion of fish choosing the rewarding arm of the T-maze and the time taken to choose the rewarding arm during the learning assessments were compared between Phase I and Phase II for each tank. Finally, the proportion of fish that chose the rewarding arms and the time taken to make the choice during the baseline and learning assessments between Tank One and Two during each phase were compared. We considered statistical significance at $p < 0.05$ (only two-tailed). We performed all statistical analysis using Microsoft Excel.

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