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The Rewarding Influence of Food: A Conditioned Place Preference Study in Zebrafish (*Danio rerio*)

Introduction

Conditioned Place Preference

Conditioned place preference (CPP) is a method that is traditionally used to assess the rewarding and aversive properties of drugs or other stimuli in laboratory animals. During a CPP test, a stimulus of interest is differentially paired with two different environmental contexts. The contexts can differ by various environmental cues, such as flooring, size, shape, and wall color. The unconditioned stimulus that is being differentially paired with the two environmental contexts generally elicits a naturally desired response, such as the rewarding properties of drugs of abuse. When the animal is introduced to the two contexts, it shows an initial preference for one side over the other. During the conditioning trials, the unconditioned stimulus is paired with the non-preferred side. Alternatively, the animal is also sequestered to the initially preferred side without the unconditioned stimulus. Following conditioning, the animal is given a choice test where it is given access to both contexts without the unconditioned stimulus. As a researcher, one is interested in seeing whether the animal spends more time in the context paired with the unconditioned stimulus than it did during the initial test. An increase in the amount of time spent in the non-preferred context is taken to indicate that the unconditioned stimulus is rewarding, and the animal learns to associate the rewarding effects with that environmental context (Bardo, 2000).

Experimenters using CPP methods generally assume that the non-preferred context becomes associated with the unconditioned stimulus through a Pavlovian conditioning process. Early theorists have argued that temporal contiguity is necessary and sufficient for learning. This means that the conditioned stimulus, or environmental context, must occur close in time with the unconditioned stimulus. There is evidence that a single element is capable of producing a CPP, however, studies have not discovered how the environmental context is neurally encoded. Recently, more attention has been given to distinguishing between elemental and configural models of learning and processing the conditioned stimulus. Elemental theories predict that each element of the paired environment is individually associated with the unconditioned stimulus. Therefore, the magnitude of place preference is equal to the sum of all of the conditioned elements that are present and processed during the test. In contrast, configural theorists suggest that the environmental context, which is comprised of multiple elements, becomes associated with the unconditioned stimulus (Bardo, 2000).

CPP vs. Other Tests

When studying the rewarding properties of drugs, many researchers use self-administration procedures instead of CPP. In contrast to self-administration, CPP is sensitive to low drug-doses and allows for results to be obtained after one drug pairing. This latter ability is one of the major advantages that CPP has over self-administration procedures and has been demonstrated with cocaine, morphine, and amphetamine. During self-administration, reliable behavior is only established after the animal gives itself repeated infusions of a drug. This repeated exposure to the drug is thought to be problematic because it likely affects receptor transduction mechanisms that are related to drug tolerance and sensitization. Using CPP, the rewarding properties of a drug can be tested after one exposure, which eliminates the risk of

inducing tolerance or sensitization. Other advantages of CPP compared to self-administration are that the animal is tested in a drug-free state and that a surgical procedure is not required (Bardo, 2000).

While there are several benefits to using CPP in the laboratory, some researchers have proposed limitations to this type of procedure. One concern is that novelty-seeking behavior could be a confounding variable. This concern is raised in CPP experiments involving rats, who are known to prefer a novel environment over a familiar one. Some have proposed that the effects of the drug might block out familiarization with the paired context, making it seem more novel when the animal is tested in a drug-free state. Studies in response to this criticism have used an apparatus with three contexts: one that is novel, one that is drug-paired, and one that is saline-paired. Using amphetamine, morphine, and apomorphine, these studies have found that rats still prefer the drug-paired environment over the novel one. However, rats also preferred the novel context over the saline-paired context. This indicates that if a drug blocks out habituation to novelty during conditioning, this could contribute to the CPP seen when the animal is tested in a drug-free state (Parker, 1992).

Another limitation of CPP is that the method does not generate dose-effect information. In order to produce a dose-dependent curve, researchers would need a group of test subjects for each point on the curve. This poses a problem since there are practical limitations on the number of drug doses and animals that can be tested. Furthermore, if CPP was used to generate a dose-dependent curve, the results would not be obtained until the final day of testing. Because of this, an experimenter would be unable to adjust the doses being tested as the study progressed. Another limitation of CPP stems from the initial preference the animal has for one context over the other. If the animal has a strong preference, this can pose a problem for the researcher.

Pairing the drug with the preferred side could fail to show CPP because of a ceiling effect, but pairing the drug with the non-preferred side may produce CPP by reducing aversion instead of establishing a true preference (Bardo, 2000).

The Role of Dopamine in Reward

It has been well established that dopamine plays a key role in reward and is mediated through the mesolimbic pathway in higher vertebrates. The mesocorticolimbic dopamine system runs from the ventral tegmental area of the midbrain to the nucleus accumbens in the striatum. In humans, stimulation of the ventral tegmental area causes the release of dopamine in the nucleus accumbens and prefrontal cortex. Dopamine release is ultimately what causes rewarding sensations of a stimulus (Ninkovic *et al.,* 2006). While drugs of abuse increase extracellular dopamine levels, studies attempting to pinpoint the receptor subtypes responsible for this effect have yielded inconsistent results. A review study by Tzchentke (1998) found that generally, it seems that the D_2 receptor is the primary mediator of increased dopamine levels.

Animal Models for CPP

Traditional CPP experiments use rodents, such as mice and rats, as test subjects because they can be bred to have specific genes that are similar to those found in humans (Tzchentke, 1998). For instance, selective inbreeding of mouse strains that show varying degrees of addiction-related behaviors has been used to correlate addiction to a few genetic polymorphisms. Transgenic mice have also been studied to correlate behaviors with known genes. While these methods are promising, they heavily rely on the candidate gene approach, meaning that only known genes can be studied (Darland and Dowling, 2001). Furthermore, it is difficult to identify genes involved in disorders because of the complexity of disorders and possible environmental factors that might influence behavior (Ninkovic, 2006). Even when the gene of interest is known,

it can take a significant amount of time to breed a large enough population of mice or rats to be studied.

Due to the problems associated with using rodents, researchers are considering other possible species that could be used in CPP studies. *Drosophila* is a species often used in genetics research because of the vast amount of information known about their genome. However, because of differences in their central nervous system compared to vertebrates, it is difficult to analyze their behavior, making them unsuitable candidates for CPP studies (Darland & Dowling, 2001). Recently, zebrafish (*Danio rerio)* have been used in biological research. Zebrafish are a small, freshwater teleost species that have a good balance between the simplicity and complexity of its organs and systems. While the nervous system of fish is simpler than in rodents, complex behaviors such as learning, addiction, aggression, and locomotion can still be studied. In addition, zebrafish reach sexual maturity within three months, and females lay eggs every morning, resulting in a large number of embryos. These factors make it easier to run large-scale screens (Gerlai *et al.*, 2000; Posthethwait, 1997). Zebrafish embryos are transparent and develop synchronously outside the mother, allowing for easy observation during development (Bilotta, 2001). They are also relatively simple and inexpensive to raise and breed (Ninkovic, 2006).

Past research has established that zebrafish are a viable vertebrate model for genetics research. Despite the obvious differences between zebrafish and humans, experimenters have found that a large number of chromosomal segments are conserved in the genomes of humans and zebrafish (Postlethwait, 1997). Using chemical mutagenesis, a large number of mutant fish can be produced, and these mutations can be mapped via techniques like bulk segregant analysis and centromere-linkage analysis. Once a mutation has been localized, insertional mutagenesis, candidate genes, and positional cloning can be used to clone a mutation of interest. During

insertional mutagenesis, a cloned DNA sequence causes genetic mutations when it is integrated into the genome. The DNA flanking the insertion is then studied to identify the mutant gene. In contrast, the candidate gene approach compares the genomic map location of a mutation to the map location of cloned genes that are expressed near tissues that have been phenotypically altered by the mutation. Finally, positional cloning identifies a DNA sequence that is near the mutation. Once a sequence has been determined, the researcher sequentially isolates overlapping DNA fragments until the gene of interest is reached (Postlethwait, 1997).

In addition to being a model vertebrate system for genetics research, a study by Darland and Dowling found that CPP can be induced in zebrafish using cocaine. At 10 mg/L of cocaine, eighty-five percent of the fish tested showed a positive change in preference. This CPP is comparable to the shift in place preference seen in experiments with mice and rats, indicating that zebrafish are a viable alternative organism for CPP studies (Darland and Dowling, 2001).

The CPP paradigm allows substances other than drugs to be tested for their reinforcing properties. Shifts in place preference have been produced using social interaction in juvenile rats, aggressive or sexual interaction in hamsters, and sucrose solution (Tzschentke, 1998). A study by Connaughton (2006) determined that a shift in place preference in zebrafish can be established using food as the unconditioned stimulus. After a single pairing of food with the initially non-preferred context, a shift in preference was observed compared to a control group that did not receive food. CPP induced by food has been found to depend on the actual consummatory act during the conditioning trials. One study found that rats that were able to eat during conditioning developed a CPP, but rats that were exposed to food they could see and smell but not eat and rats that were fed after the conditioning trial in their cages did not develop a CPP (Maes and Vossen, 1993).

The purpose of this study is to replicate the findings from the earlier CPP study in zebrafish using food as the unconditioned stimulus. Based on reviews of CPP experimental designs, a few methodological changes will be implemented in this study in hopes of observing a stronger shift in place preference. I hypothesize that a stronger shift in place preference will be observed when food is used as the conditioned stimulus compared to control conditions.

Materials and Methods

All zebrafish were obtained from local suppliers, such as Petsmart or PETCO Stores. Test subjects were randomly selected from a stock tank and isolated into opaque ~1-liter holding containers. Fish were given 24-hours to acclimate to their holding containers before the experiment began. These containers were kept in a 27°C water bath when the fish were not involved in the experiment.

During the experiment, a 5-liter, rectangular tank was used. This tank was divided down the center with one of two removable dividers. The first divider had several smaller slits that allowed water flow, but prevented the fish from passing between sides (Divider 1). The second divider had two 1" x 1" square openings that allowed the fish to have unrestricted access to both sides of the tank (Divider 2). In this experiment, a biased tank design was used, meaning that the tank is designed in such a way that the animal does exhibit an unconditioned place preference for one side over the other. If a biased tank design is to be used, it is important to set up the environmental cues so that the animal has a clear initial preference for one context, but the aversion to the non-preferred side can be overcome by the rewarding properties of the unconditioned stimulus. For zebrafish CPP experiments, a review study by Ninkovic (2006) found that the best results were obtained when the contrast between the two sides is decreased. To do this, the author suggests that a tank has a dark brown compartment and a lighter

compartment that is white with two black spots. In this experiment, we implemented Ninkovic's suggestion, which is one point where this study differs from previous studies that used strictly black and white backgrounds.

For this experiment, five fish were assigned to the experimental group and five fish were assigned to the control group. After being numbered and placed into the holding beakers, experimental subjects were not fed unless during the conditioning cycles. The experiment always began between 10 AM and 11 AM, and the fish were always placed in the testing tank for fifteen minutes. On the first day of the experiment, the fish were placed on the left (brown) side of the tank with divider 2. This day served as a pre-test to determine each fishes' initial preference and was documented using a digital recorder. On the second day, the fish was placed on its initially non-preferred side with divider 1. Subjects in the experimental group were fed two large flakes of Tetramin flake food, which was ground up using a mortal and pestle. On the third day of testing, each fish was placed on its initially preferred side without food. Days two and three formed the conditioning cycle, which was repeated two more times over days 4-7. On the last day of testing, the fish was placed on the left (brown) side of the tank again with divider 2. This day served as a post-test to determine any shifts in place preference that may have resulted. The post-test was also documented with a digital recorder.

The procedure was the same for the fish in the control group, except that they were not fed during the experiment. Instead, these fish were fed two large flakes of ground up Tetramin flake food daily upon being returned to their holding containers. During the conditioning cycles, the control fish were not fed food when they were sequestered on the initially non-preferred side. Following the post-test, all fish were anesthetized with Tricaine and preserved in 4% paraformaldehyde solution for later analysis.

For all fish, recordings of the pre- and post-tests were played back to measure the amount of time spent on each side of the tank. To measure changes in place preference, the amount of time spent during the pre-test on the initially non-preferred side was subtracted from the amount of time spent on that side during the post-test. Changes in place preference were analyzed for each individual fish, as well as by calculating the averages for the experimental and control groups. A two-proportion z-test was used to determine the significance of the results. Results were considered significant if $p \leq .05$.

Results

Experimental Group

After performing the pre-test, it was determined that three fish in the experimental group preferred the brown side of the tank, while the other two fish preferred the white side of the tank. All fish in the experimental group spent a portion of the fifteen minute period on the non-preferred side. The time on the non-preferred side ranged from 102 to 393 seconds out of a possible 900 seconds (Figure 1a). After the three cycles of conditioning, a shift towards the non-preferred side was seen in 80% of the fish in the experimental group. Among the fish who displayed a shift in place preference, the increase in the amount of time spent on the initially non-preferred side ranged from 72 to 424 seconds (mean = 184.25 seconds) (Figure 1b).

Figure 1a: Experimental Group Pre-Test

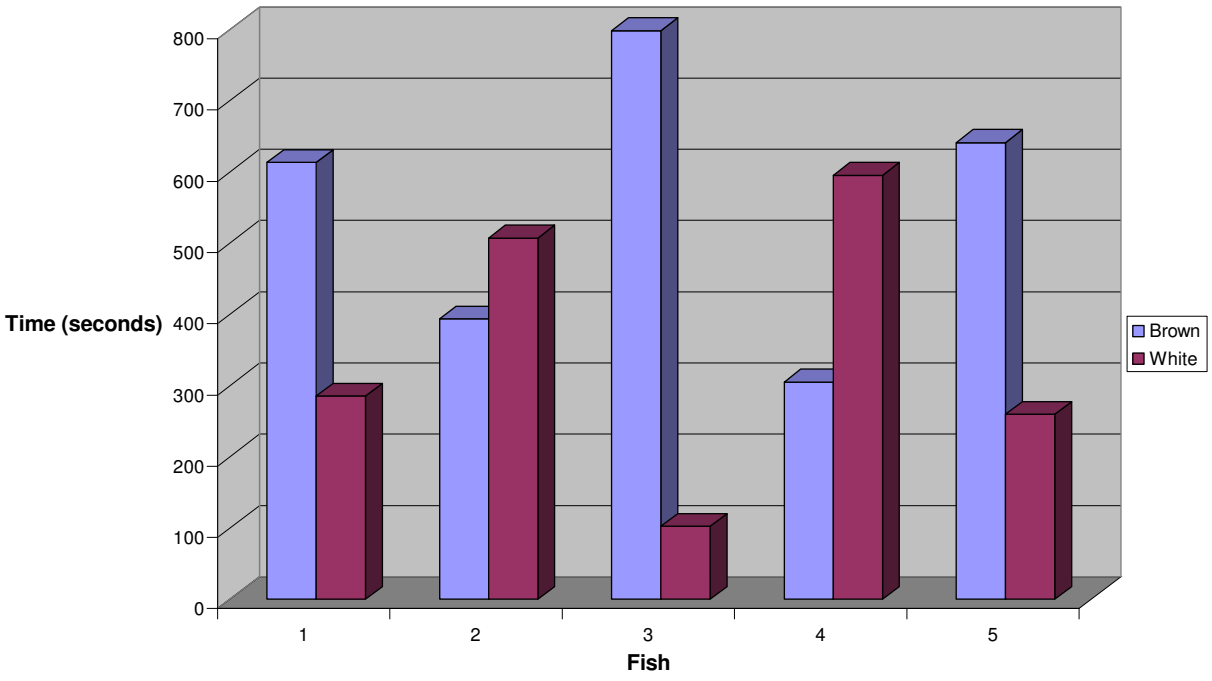

Figure 1a depicts the amount of time spent in each of the environmental context by the fish in the experimental group during the pre-test. The fish were observed for 15 minutes (900 seconds). The blue bars depict the amount of time spent on the brown side of the tank, and the purple bars depict the amount of time spent on the white side. Three fish (Fish 1, 3, and 5) initially preferred the brown side, while two fish (Fish 2 and 4) initially preferred the white side.

Figure 1b: Experimental Group Post-Test

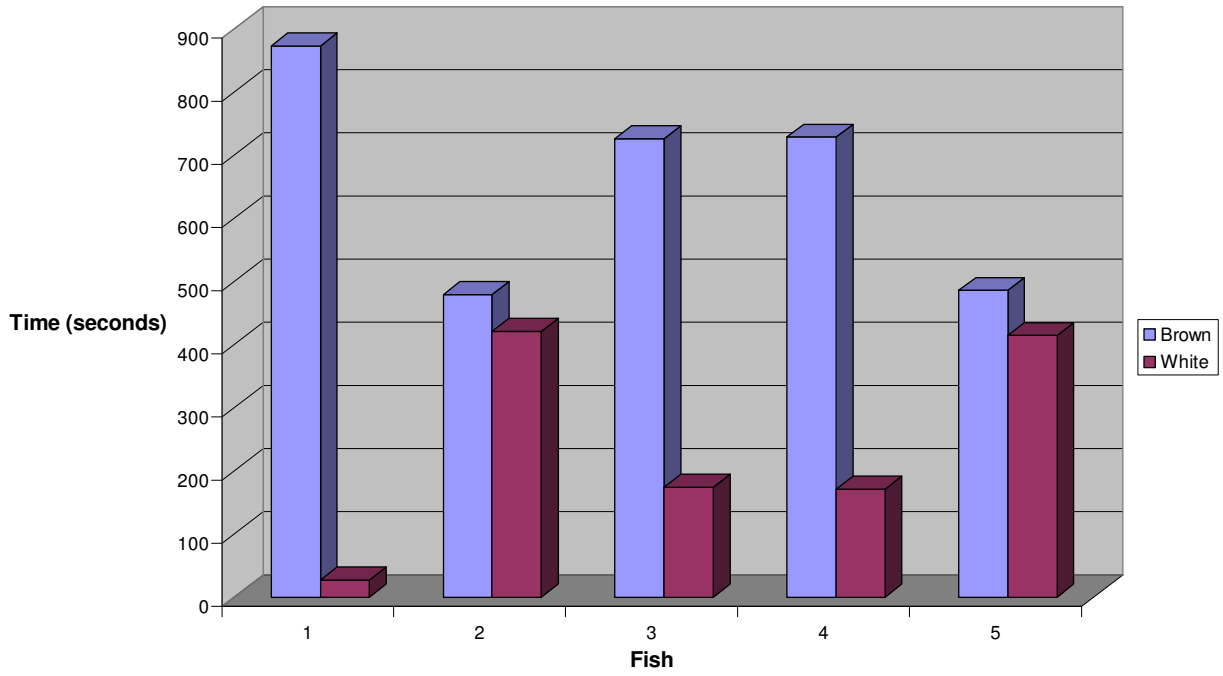

Figure 1b depicts the amount of time each fish in the experimental group spent in each of the contexts during the post-test. Fish 2, 3, 4 and 5 all showed shifts towards the initially non-preferred side of the tank.

Control Group

In the control group, four fish initially preferred the brown side of the tank, and only one preferred the white side. Four of the five fish spent some time on the non-preferred side during the pre-test, ranging from 57 to 416 seconds (Figure 2a). After the three cycles of conditioning, where food was not paired with either environmental context, only two fish, or 40% of the control group, displayed a shift towards the initially non-preferred side. The increase in the amount of time spent on the non-preferred side ranged from 283-473 seconds (mean = 378 seconds) (Figure 2b).

Figure 2a: Control Group Pre-Test

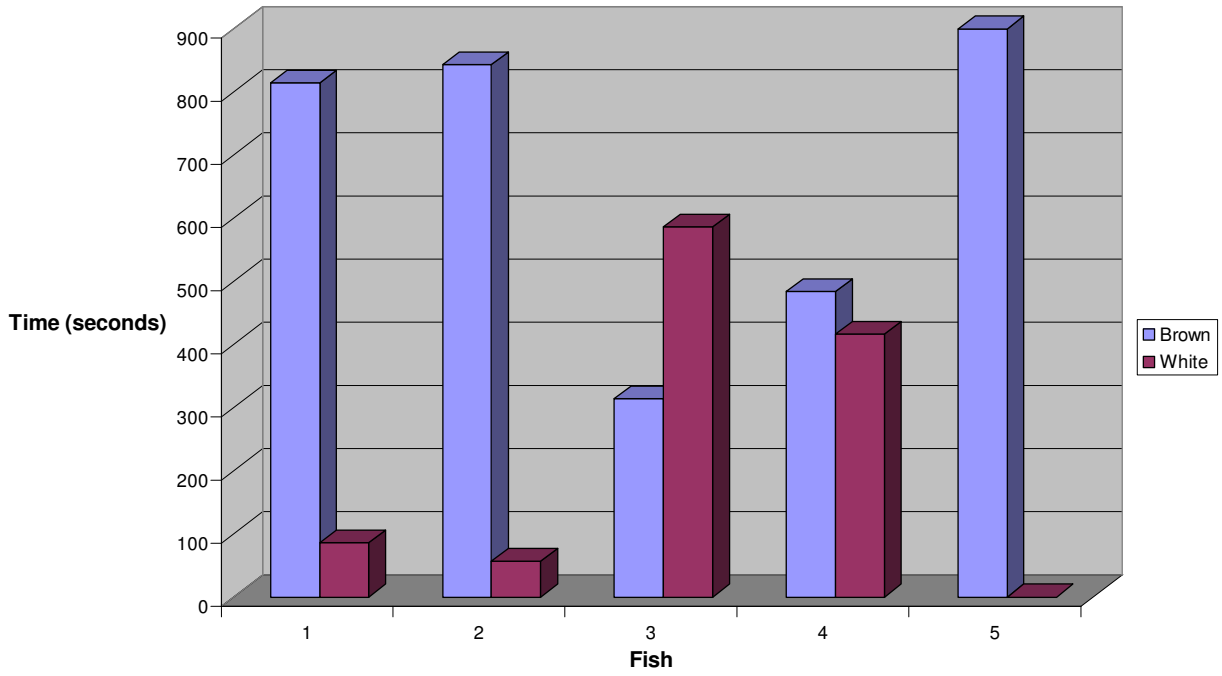

Figure 2a depicts the amount of time spent in each of the environmental context by the fish in the control group during the pre-test. Four fish displayed an initial preference for the brown side of the tank (Fish 1, 2, 4, and 5), while only one fish displayed a preference for the white side (Fish 3).

Figure 2b: Control Group Post-Test

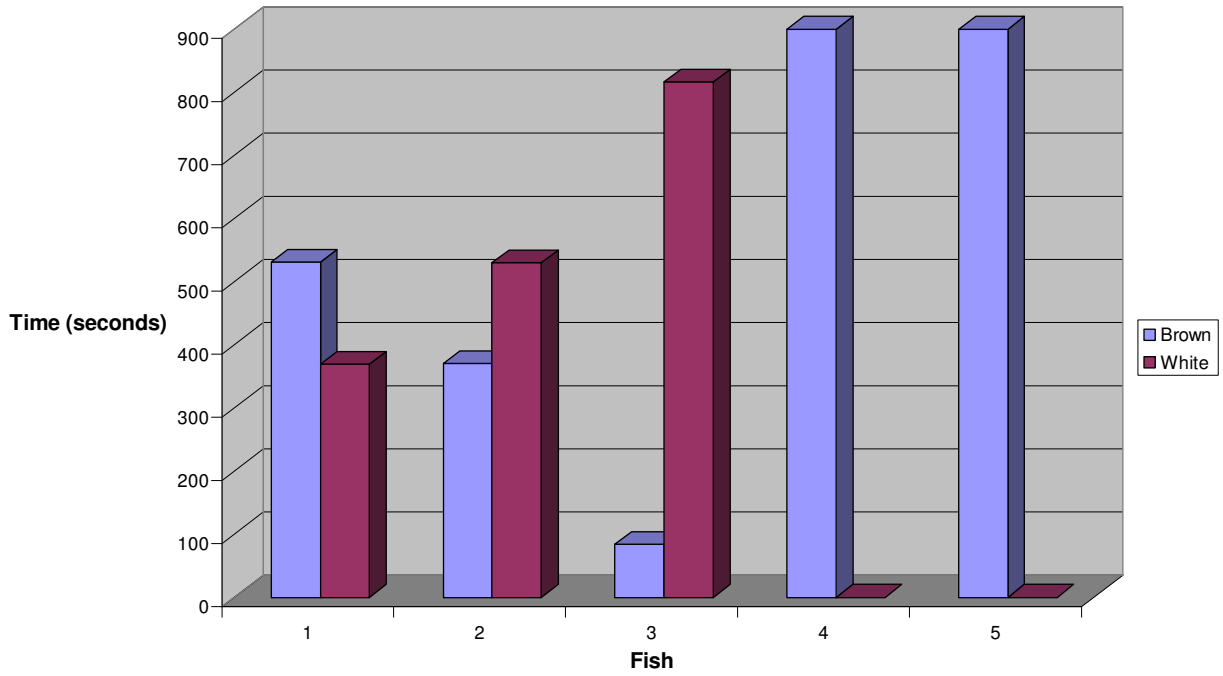

Figure 2b depicts the amount of time each fish in the control group spent on each side of the tank during the post-test. Fish 3 and 4 displayed shifts towards the initially non-preferred side of the tank.

Statistical Analysis

Once the data was analyzed, a statistical test to compare two population proportions was used to determine the significance of the results. When this test was completed, we found that the difference between the numbers of fish in each group showing a shift in place preference was significant ($p < .00003$). A t-test was used to determine that any differences between the groups in the degree of shift in place preference were not significant ($p = .0752$).

Discussion

The primary findings from this study demonstrate that preference for the initially non-preferred side increases after three cycles of conditioning. The 80% shift in the experimental group towards the non-preferred side compared to only a 40% shift in the control group confirms the original hypothesis that a larger shift would be observed when food was used as the

conditioned stimulus. These results strengthen previous claims that food can be used as a conditioned stimulus in a similar manner to drugs of abuse. While we were unable to test the concentrations of dopamine in the brain, the shift seen in the experimental group suggests that food is activating some reward pathway in the brain.

One unexpected result that was obtained during this experiment was the 40% shift observed in the control group. The previous study using food did not observe a shift in the control group, and I am unsure of why I saw a change. It is possible that procedural differences could account for this inconsistency. In the previous study, subjects were only observed for two minutes, whereas I observed my subjects for fifteen minutes. When I only analyzed the first two minutes of the data, only one fish, or 20%, of the control group displayed a shift towards the non-preferred side. If the fish in the previous experiment had been observed for a longer period of time, it is possible that a shift in place preference in the control group may have been observed. Another possibility is that the shift observed in the control group could have been a random occurrence that was magnified by the small sample size used. It will be important to replicate this study in the future with more subjects to determine whether this finding in the control group warrants further investigation.

While an 80% shift was observed after three cycles of conditioning with food in this study, a two-proportion t-test revealed that this was not statistically significantly different from the ~70% shift observed in the previous study that only used one conditioning cycle ($p = .28035$). Traditionally, CPP experiments using rodents require at least three conditioning cycles in order to obtain reliable results. However, it seems that comparable results can be obtained in zebrafish after only one cycle. This suggests that zebrafish may be more sensitive to conditioning than rodents. While this would need to be replicated with more subjects, this finding could have

important implications for researchers designing future CPP experiments. If zebrafish are in fact more sensitive to conditioning, then less testing time would be required for each fish. This could translate to more time efficient experiments or the ability to test more subjects.

There were a few areas of this study that could be improved upon in the future. As I have already mentioned, the sample size in this study was small. The previous experiment included twenty-four subjects, but because I was observing the fish for a longer period of time, there were some limitations on the number of subjects I could watch. If this experiment is replicated in the future, it will be important to plan for the study to be spread out over a longer period of time so that more fish can be used. Another area of improvement in this study is in the experimental tank design. Many CPP experiments that are testing rodents use three-compartment conditioning boxes. In this type of apparatus, the third chamber serves as a neutral compartment that connects the two conditioning compartments (Tzschentke, 1998). While I attempted to control for confounding variables by placing all of the fish initially on the brown side of the tank during the pre- and post-tests, it is possible that this may have had an effect on preference. If a three-compartment conditioning box was used in the future, the fish would have the initial choice of picking the environment, instead of the environment being chosen by the experimenter.

Conclusions

The results from this study support the suggestion that zebrafish are a viable model for CPP experiments. Furthermore, this study demonstrates that food can be used as a rewarding stimulus in place of drugs of abuse. The results that were obtained in this study are significant because they suggest that zebrafish may be a superior model for CPP compared to rodents due to possible increased sensitivity to conditioning. Replications of this study are needed before one would be able to determine whether or not this is true.

There are a few ways that this study could be expanded upon in the future. From the results of this study, it is impossible to determine whether the CPP that was induced in zebrafish occurred because the food was rewarding or because of motivational behavior. In order to determine whether the food was rewarding, it would be necessary to look more closely at the biochemistry and anatomy of the zebrafish brain. A study by Bretaud *et al.* (2007) that looked at choice behavior for morphine in zebrafish has proposed that the connection between the posterior tubercular region of the ventral forebrain and the subpallium, which is thought to be equivalent to the striatum in humans, could be important in mediating reward. The findings of Bretaud's study are significant because they provide possible brain regions in zebrafish that may be analogous to the mesolimbic reward pathway of humans. For future research using zebrafish as a model organism for reward in higher vertebrates like mammals, these brain regions may play a key role in our understanding of the reinforcing effects of drugs and other stimuli.

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