SHOALING DIFFERENCES IN ZEBRAFISH, *DANIO RERIO,* AND YELLOW GLOFISH, GENETICALLY ALTERED *DANIO RERIO*

A Report of a Senior Study

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Abstract

Glofish are the first genetically modified pets that are available to the public, and it is important to look at their social behavior. The shoaling behavior of the zebrafish and genetically modified zebrafish, yellow Glofish, was observed by calculating how much time the fish spent near each other. This was done in four separate shoaling tests by calculating the amount of time either a Glofish individual or zebrafish individual spent in a 10 minute, 600 second, period when given the choice between a shoal of phenotypically similar fish, an empty compartment, or a shoal of phenotypically different fish. The results demonstrated that all of the four test groups showed a significant difference when compared to the null hypothesis that the individual fish would spend 300 seconds on each side. Therefore, both zebrafish and yellow Glofish preferred to shoal with fish that were phenotypically similar. The overall results from the t-test and the ANOVA test proves that zebrafish discriminate but Glofish do not. Other studies have shown that zebrafish and red Glofish spend an equal amount of time near each other, proving that color is important in shoaling for both zebrafish and Glofish.

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Acknowledgements

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CHAPTER I

INTRODUCTION

An important question to answer is how genetically modified animals are different from the animals they were derived from. Glofish are genetically modified zebrafish, or genetically modified *Danio rerio*, that have been given a fluorescent protein to give them a glowing and colorful appearance. These Glofish were originally created to test water for toxins, and they were predicted to glow when they found the presence of a toxin. Glofish became the first genetically modified pet sold to the general public in 2003 (Snekser et al. 2010). They can be found in many pet stores in various colors such as red, yellow, green, blue, and purple. It is possible that eventually other animals would also become genetically modified, but it is important to see if this modification might be harmful to the animals or to the environment. Glofish are considered to be a different strain from zebrafish. In this study behavior will be used to look at differences and similarities between zebrafish and Glofish. More specifically, shoaling behavior will be used to look at the similarities between zebrafish and Glofish.

Zebrafish have been used over the years for research in genetics and developmental biology, but they have only recently been studied in animal behavior. One study done by

Xia et al (2009) used zebrafish behavior as an indicator or biomarker for detecting harmful chemical pollutants such as nonylphenol, which can be found in aquatic ecosystems, and is of high environmental concern along with ethanol (Kurta and Palestis 2010). The zebrafish is a small tropical fish species that lives in groups in which dominance hierarchies readily form between individuals (Filby et al. 2010). Zebrafish are a highly social species that prefer to swim in groups, an aggregation behavior termed shoaling (Saverino and Gerlai 2008). Since zebrafish are naturally social, they are usually found in shoals of two to ten. They are a native fish in freshwater streams and lakes of India, Pakistan, and Bangladesh (Vital and Martins. 2011). Observations that have been conducted suggest that zebrafish seem to prefer still or slow moving, slightly alkaline water of relatively high clarity (Lawrence 2007).

Zebrafish are characterized by a black and white striping pattern running horizontally across their bodies similar to that of a zebra as shown in figure 1. Yellow Glofish also have a striping pattern running horizontally through their bodies, but they have a much brighter coloration than zebrafish. Glofish are known to actually glow in the dark with a use of a back light. The ability to glow is part of their genetic modification, and zebrafish do not posses this ability.

Figure 1. Phenotypic differences between zebrafish (left) and yellow Glofish (right).

Schooling is an important social behavior in many types of fish. Zebrafish form schooling groups, which is a behavior known as shoaling (Miller and Gerlai 2007). This type of behavior is very important for the survival of zebrafish for many reasons. Shoaling is thought to provide the individual fish with multiple benefits, including access to mates, efficient foraging and defense against predators (Buske and Gerlai. 2011). Shoaling is important for foraging and protection, but it is also shown to be important in information transfer, learning and other expression of behavior (Moretz et al. 2007). Shoaling is thought to be under a double constraint, as a shoal density is inversely related to foraging efficacy but directly related to predator avoidance (Maximino et al. 2010). Zebra fish have strong shoaling preferences influenced by both visual and chemical cues (Vital and Martins 2011). Therefore, shoaling is important for the survival in zebrafish and Glofish.

In fish, shoaling behavior is known to be influenced by characteristics such as body coloration, striping pattern, body shape, and size (Snekser et al. 2010). It has been shown that zebrafish shoaling preferences are influenced by coloration that is similar to their own, such as similar stripes or the same amount of yellow coloration (Miller and Gerlai 2007). Sex and phenotype can also impact schools and behavior in schools (Snekser et al. 2010). The individual fish can receive many advantages including the "confusion effect." According to this effect model, a predator faced with a group of similar fish has difficulty targeting an individual to attack. This is also related to the "oddity effect," which predicts that physically distinct, or odd, individuals within a shoal are more likely to be targeted by predators. Therefore, an individual's ability to recognize and associate with similar fish should provide a selective advantage. Previous studies have also been shown that fish

prefer to shoal with phenotypically similar individuals (Snekser et al. 2006). In studies on the confusion and oddity effects, a number of phenotypic attributes have been shown to affect shoaling preferences for a large variety of fish species (Snekser et al. 2010). Some of these features would include body size, parasite infection such as black spots on the body, body shape, body coloration, and stripe pattern (Snekser et al. 2010). Therefore, as it is predicted fish are capable of discriminating between potential shoal mates based on certain phenotypic features, and they typically school with fish that are similar in appearance (Snekser et al. 2010). The fish could then be able to benefit from being in the school without as much fear of predators. Fish in a school tend to maintain a certain neighbor distance in certain conditions (Larsson 2009).

The development of the octavolateralis system, which is known as a sense organ in fish that responds to changes in the environment, in fish ancestors created the phenomenon of sensory reafference associated with the fish's own locomotion (Larsson 2009). In fish species living and moving in groups, there is potential to produce complex pressure waves and other water movements interfering with the octavolateralis (Larsson 2009). Therefore, according to Matz Larsson (2009), the development of the octavolateralis system may have started from the evolutionary development of synchronized group locomotion, or shoaling.

According to Snekser et al, the development of transgenic fish creates an opportunity for studying the importance of body coloration both socially and reproductively (Snekser et al. 2010). This is important because fish have been known to discriminate between potential shoal mates based on phenotype and body coloration (Snekser et al. 2010). For example, black and white mollies choose shoal-mates of similar coloration such as black

mollies shoaling with other black mollies as opposed to dalmatian mollies, or white mollies (Snekser et al. 2010).

Social experiences and social environments can also influence the shoaling behavior in fish. Social status can affect an individual's access to food, mates, and shelter and has consequences for the physiology of individuals and their health status (Filby et al. 2010). Even in social groups, zebrafish can become aggressive (Moretz et al. 2007). Zebrafish also have hierarchies in their social groups based on dominance (Filby et al. 2010). Social experiences have been shown to influence fish antipredator behavior, migration, foraging and mate choice (Moretz et al. 2007). Therefore, Social experiences can shape an individual's ability to recognize similar mating and shoaling partners. According to Moretz et al (2007), in shoaling fishes, the social environment may play a substantial role in the development and expression of behavior in individuals because of their frequent contact with other group members. The environment that zebrafish live in also proves to be an important part of survival and development. In novel environments, zebrafish swim to tank bottoms and dark backgrounds. This behavior allows them to hide in a certain part of their environment to protect them from predators (Sackerman et al. 2010). One study done by Kristine von Krogh (2010) proved that the behavior and physiology of zebrafish was much greater in an enriched environment as opposed to a barren environment. In zebrafish early social environment appears to be important in forming some association preferences such as horizontally striped or white fish (Moretz et al. 2007). However, other behavior patterns such as the startle response, surface orientation, and tendency to remain near a shoal, seem to have a strong genetic basis (Moretz et al. 2007). The study done by Moretz et al (2007) shows that both social behavior and social environment are

important. They showed that many fish have the ability to identify individuals and kin in shoals and can exhibit less aggression to kin or familiar individuals (Moretz et al. 2007). Therefore, they have been shown to be less likely to shoal in close proximity of unfamiliar fish.

One experiment was done by Snekser et al. (2010) to look at shoaling preferences of male and female mutant zebra fish, wildtype zebrafish, and pearl zebra fish. They found in their experiment that when given a choice of shoalmates, wildtype zebrafish of both sexes showed no discrimation between the two strains, but they still preferred to shoal with the wildtype zebrafish (Snekser et al. 2010). However, for both the mutant and pearl zebrafish, the shoalmate preferences revealed that males showed no discrimination, while mutant females preferred shoals of their own strain (Snekser et al. 2010).

There are several controversial issues of genetically modified animals such as Glofish. Glofish could potentially harm the ecological environment if they were released. Therefore, environmental safety is a concern. Aquarium fish can often be introduced into local recipient waters to which the fish are not native. Fish are both difficult to contain and highly mobile, and transgenetic fish can become feral and invade native ecosystems (JHA P. 2010). It has become likely that Glofish will be released into nature, just like wildtype zebrafish have been found outside captivity in California, Florida, Connecticut, New Mexico and Wyoming (Cortemeglia et al. 2008). Transgenic organisms are similar to those of invasive exotics because they can present similar environmental concerns. This is because transgenic organisms can have novel or enhanced abilities relative to their wild type counterparts. Therefore, transgenic organisms might threaten the survival of wild type counterparts as well as other species in the community (JHA P. 2010).

Some scientists have hypothesized that the genetically modified zebrafish will not persist in nature because they will be preyed upon more often since they have such bright colorations (Cortemeglia and Beitinger 2006). Yet the bright coloration could also protect them because they are also not preyed upon immediately by predators in their native range (Cortemeglia and Beitinger 2006). These two controversial hypotheses have not yet been tested in the wild. After the experiment done by Cortemeglia and Beitninger to see if Glofish or zebrafish would be preyed on by native predators, it was concluded that zebrafish and Glofish would be equally preyed on (Cortemeglia and Beitinger 2006). Therefore, it was shown that the Glofish do not have specific traits that would allow them to have greater predator avoidance or greater predator susceptibility (Cortemeglia and Beitinger, 2006). However, a recent study was done by Cortemeglia et al (2008) that examined zebrafish and Glofish response to a decrease in water temperature. They concluded that a decrease in temperature during the cold months of the year would be the most likely cause of death for Glofish that may be released in nature, as opposed to predation or starvation (Cortemeglia et al. 2008). The release of Glofish is still a concern, but recent studies are beginning to make Glofish not seem to be as big of a threat as originally thought.

Zebrafish are considered to be fairly complex fish by many scientists, which is one of the reasons why they are used as models in various studies in genetics, developmental biology, and behavior studies. Therefore, the husbandry of zebrafish should be important for their care. Experimental evidence shows that the degree of genetic relatedness of individuals in shoals of adults is unknown, although experimental evidence indicates that an individual's preference to associate with siblings switches to avoidance after sexual

maturity (Lawrence 2007). The life span for zebrafish has been documented in captivity to exceed five years (Lawrence 2007). Zebrafish can tolerate a wide range of temperatures but the recommended temperature is 28.5°C (Lawrence 2007). The desired pH for zebrafish has been suggested to be between 7 and 8 (Lawrence 2007). The correct care of zebrafish is extremely important for their well being, and if they are cared for in the right conditions and in the right environment they may live more than five years.

In this study, zebrafish and Glofish were compared to see if they shoal with individuals of a different phenotype or if they will only shoal with members of their own phenotype. This is beneficial to see if genetically modified fish are different or similar to the fish that they were modified from. The study specifically examined if Glofish will shoal with others of the same or different phenotype if given the choice. For example, this study observes the preference of zebrafish and Glofish in certain shoaling differences such as phenotypically similar shoals, phenotypically different shoals, or empty containers. The study was created to determine if an individual Glofish will prefer to shoal with a group of three zebrafish, a group of three other phenotypically similar Glofish. The Glofish will be studied to see if fish of one phenotype avoid shoaling with fish of another phenotype or if they prefer to shoal with fish displaying a certain phenotype or if they simply choose to swim alone without a shoal. The study will also look to see which phenotype an individual zebrafish would prefer to shoal with such as a group of three other phenotypically similar zebrafish, or a group of three Glofish of one color, or simply swim alone without a shoal.

A similar experiment was previously done by Saverino and Gerlai (2008) to look at how a zebrafish would respond to four different types of stimulus fish made from

computer images. The first fish they used were their own conspecifics, which simply meant that the fish are a similar species, that look similar to them. The second fish used in the experiment was another similar species fish whose color is slightly different (color variant). The third fish was a fish that was a very different species but phenotypically similar. The fourth fish was a fish that was a similar species and it looked similar to the zebrafish but it is not a shoaling fish (Saverino and Gerlai 2008). They noted that a greater similarity among group members has been found to reduce predation under natural conditions because it minimizes phenotypic oddity (Saverino and Gerlai 2008). According to Saverino and Gerlai (2008), this social understanding of zebrafish could eventually help to explain the social behavior in other animals and humans. They also noted that color has been found to be an important factor affecting choice behavior in fish (Saverino and Gerlai. 2008). They found that even though red colored images of zebrafish, which are not naturally found in nature, were not avoided, the unaltered images of zebrafish were more significantly preferred (Saverino and Gerlai. 2008). However, zebrafish had a strong preference for yellow colored images. The reason for this was probably because vividly colored zebrafish exhibit more yellow coloration, and therefore it is possible that the yellow colored computer image showed a trait that was interpreted by the zebrafish to indicate healthy or reproductively mature fish (Saverino and Gerlai 2008).

One study was done by Sackerman et al. (2010) to compare the behavior of two different strains of zebrafish and a Glofish strain to see if they preferred to stay close to the bottom of the tank and to see if they preferred light or darker areas of the tank. After the first experiment they found that all three strains preferred to stay in the bottom two

thirds of the tank, which showed that their behavior was similar (Sackerman et al 2010). They predicted that the fish would prefer to stay in the darker areas as a way to hide from predators (Sackerman et al 2010). After the light and dark test, they concluded that all the strains behaved in the same way towards the light and dark parts of the tank (Sackerman et al 2010). These tests showed that the three different strains all had similar behavior based on the way they reacted to both of the tests.

Traditionally, fish that are within four body-lengths of each other, which is about 16 cm for zebrafish, are considered part of the same shoal (Miller and Gerlai. 2007). This method is used to measure the distance between close neighbors. The school will typically stay stable. However, with the presence of food in the tank, the zebrafish shoal may spread out and not be as close as 4 body lengths (Miller and Gerlai. 2007). According to Miller and Gerlai (2007), two different methods are usually used when looking at fish behavior. One method is to look at the shoaling behavior by measuring the preference of a test fish placed in a central compartment of a test tank flanked by two adjacent compartments which contain a shoal of conspecifics or are empty (Miller and Gerlai. 2007). This method has been shown to show shoaling preferences of fish but it also has an artificial nature, while the other method allows the behavior of the fish to be examined in a more freely moving shoal (Miller and Gerlai. 2007). The fish in the freely moving shoal would have no barriers between them, but would instead be allowed to swim anywhere in the tank along with having access to the other fish. Both methods have been used with studies similar to this one. However this study will use the method that requires the use of compartments to test the preference of an individual to form a shoal when given the choice between two phenotypic ally different groups.

This study was specifically testing the hypothesis that when an individual is given the choice between a school of zebrafish, a school of Glofish, or an empty compartment, it will choose to school with the fish that are most phenotypically similar to itself. This was predicted based on fish choosing to shoal with a group of similar fish to protect the individuals from predation because individuals that are physically distinct can be more likely to be targeted by a predator. This prediction was based on the confusion effect, which will protect the individual fish from predators. This is also related to the "oddity effect," which predicts that physically distinct individuals within a shoal are more likely to be targeted by predators. Therefore, an individual's ability to recognize and associate with similar fish would provide an advantage against predators.

This study determined how zebrafish and Glofish behave together in shoals, which could help to determine how behaviorally similar or different they are. Since they are genetically different strains of zebrafish, it is important to see if they have the same behaviors. However, if the Glofish's behavior was proven to be very different or aggressive, it could harm the environment if the Glofish were released. This could also show how genetically modified animals are different from the animals they are derived from, which would become important if other animals eventually become modified.

CHAPTER II

MATERIALS AND METHODS

Adult fish were obtained from a commercial supplier, PetSmart in Maryville, TN. A total of twelve zebrafish, and twelve yellow Glofish were obtained. They were then housed separately in shoals of six in ten gallon tanks. Therefore, there were two shoals of six zebrafish and two shoals of six yellow Glofish. The shoals were formed to separate the fish so that during the experiments the fish would be observed with fish from the opposite group. Therefore, the tests would be random, and the fish would not be observed with fish that they were already familiar with. All four of the tanks containing the fish were covered on three sides with paper to visually isolate the fish from the other groups. Each tank contained its own water filter, to keep the water clean and oxygenated. The tanks were kept under lighting of 12 hours of light and 12 hours of dark cycle at 23°C. All of the fish were fed the same amount of commercial flake food once a day.

For the shoaling experiments, a ten gallon test tank was divided by creating three separate compartments in the tank. As shown in Figure 2, the compartments were separated by the use of two 3000 mL beakers in which the shoals were house during the experiments. The individual was then allowed to swim freely in the tank around the

beakers. A line of paper was taped directly on the center of the glass. The line separated the tank into two sides, but the individual fish was still allowed to swim freely across the line in the tank. The amount of time the individual fish spent on each side was recorded. It was assumed that if there was no preference for either shoal, than the individual fish would spend 300s (50% of their time) on each side of the tank.

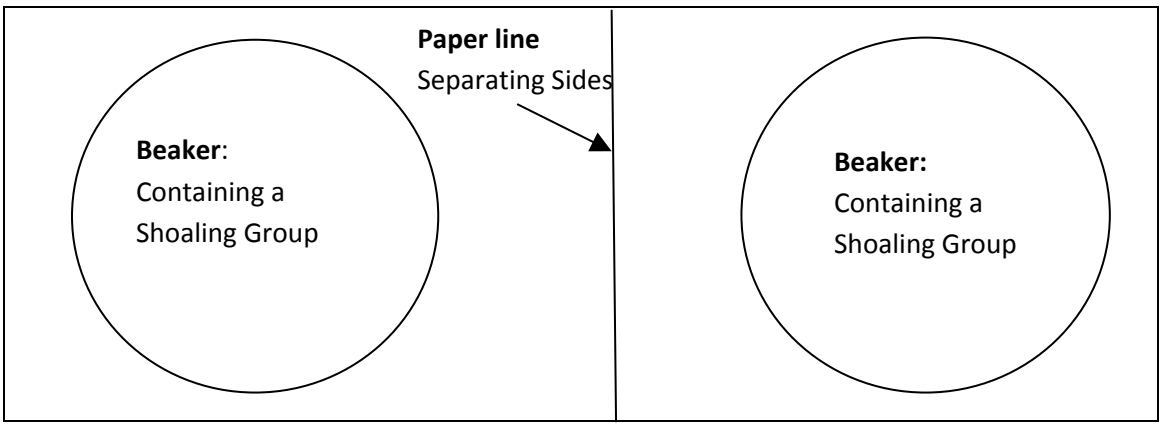

Figure 2. Ten gallon test tank design exhibiting the two beakers on both sides along with the paper line in the middle separating the sides, while the individual fish is allowed swim across the line.

Prior to each shoaling experiment, the individual test fish used was placed in the center and allowed to swim freely around the tank while the shoals of stimulus shoaling fish were placed in beakers. The individual test fish and shoaling groups of three were randomly chosen for each trial. All fish were given a period of 30 minutes to acclimate. An equal number of individual trials were run with the stimulus shoals on the left and right end of the tank compartments for each test to allow for more randomized trials. Shoaling experiment sessions were recorded during a period of about a month. A total of

four different shoaling experiments were completed, and each included fifteen separate trials. Each trial session consisted of a ten minute period, or six hundred seconds. During that 600 second period of time, the amount of time each individual test fish spent on each side of the tank was recorded.

The experimental design exhibiting the four different shoaling experiments is represented in table 1. Each of the four different experiments consisted of an individual that was allowed to swim freely, which is listed in the second column, while each end beaker either contained the test shoals. Each test shoal either consisted of a shoal of three randomly chosen zebrafish, a shoal of three randomly chosen Glofish, or it was simply left empty.

Table 1. Shoaling experimental design exhibiting the four different shoaling groups used.

Shoaling Experiment Number	Individual Test Fish Swimming freely in the tank	Shoal 1 in end beaker	Shoal 2 in end beaker
1	Wildtype Zebrafish Individual	Wildtype Zebrafish Group	Empty
2	Wildtype Zebrafish Individual	Wildtype Zebrafish Group	Glofish Group
3	Glofish Individual	Glofish Group	Empty
4	Glofish Individual	Glofish Group	Wildtype Zebrafish Group

In the first group of shoaling experiments, the preference of an individual test zebrafish was examined. In shoaling experiment 1, an individual test zebrafish randomly chosen from one group was placed in the middle of the tank and allowed to swim freely,

while a shoal of three randomly chosen zebrafish from the other group was placed in one of the beakers. The other beaker in the tank was simply left empty. Therefore, shoaling experiment 1 was used to see if the individual test zebrafish would prefer to stay near the shoaling group of zebrafish or if the single test zebrafish preferred to stay alone. Shoaling experiment 2 was used to look at an individual test zebrafish's preference for either shoaling with a group of other zebrafish or a group of Glofish. This experiment was done by placing a group of three randomly chosen Glofish in one of the beakers and a group of three randomly chosen zebrafish in the other beaker.

The second group of shoaling experiments focused on Glofish preference. Shoaling experiment 3 observed the preference of an individual Glofish randomly chosen to be alone or to shoal with a group of Glofish. This experiment was done by leaving one beaker empty and placing a shoal of three randomly chosen Glofish in the other beaker. Shoaling experiment 4 was used to examine the preference of a Glofish when given the choice between shoaling with a group of zebrafish or a group of Glofish. This test was done by placing a randomly chosen individual Glofish in the center of the tank to observe the Glofish's preference. Then a randomly chosen group of three shoaling Glofish from the other group was placed in one beaker, and a group of three randomly chosen zebrafish were placed in the other beaker.

Two different statistical analysis were used in the four group experiments. The first was based on the expectation that if there was no preference for either shoal, than the test individual fish would spend 300s, 50% of their time, on each side of the test tank. Therefore, a comparison of the collected times for each of the four group experiments was compared to the null expectation of 300s on each side using a one-sample t-test for

each test. The other statistical analysis used was an ANOVA one way post-hoc Tukey test. The ANOVA test was used to compare each the four separate shoaling experiments with each other, to examine differences among the experiments. For example, if there was a difference between shoaling experiment 1 and shoaling experiment 2.

CHAPTER III

RESULTS

All four of the shoaling experiments were repeated fifteen times to determine a mean amount of time spent on each side of the tank. This meant that the amount of time spent on one side was compared to other mean amount of time spent on the other side. It was also compared to the null expectation that the fish would spend an equal amount of time on each side, which would be an average of 300 seconds.

In shoaling experiments 1 and 3, in which test fish were given a choice between a shoal of three fish and an empty compartment, both individual zebrafish and Glofish test fish always exhibited a significant preference for being near the shoal of fish as opposed to being alone. Both zebrafish and Glofish individuals in shoaling experiments 2 and 4 exhibited preferences for being near shoals of the same phenotypic similarity. However, both zebrafish and Glofish individuals preferred to spend more time near shoals of different phenotypes as opposed to spending time near an empty compartment.

Figures 3 and 4 represent the mean preferences of the test individuals to the shoaling groups the individuals were exposed to. Figure 3 exhibits the zebrafish preference with shoaling experiments 1 and 2. Figure 4 exhibits the Glofish preference with shoaling experiments 3 and 4. Both figures exhibit the shoal of zebrafish as a black color, a shoal of Glofish as a gray color, and an empty compartment as a white color.

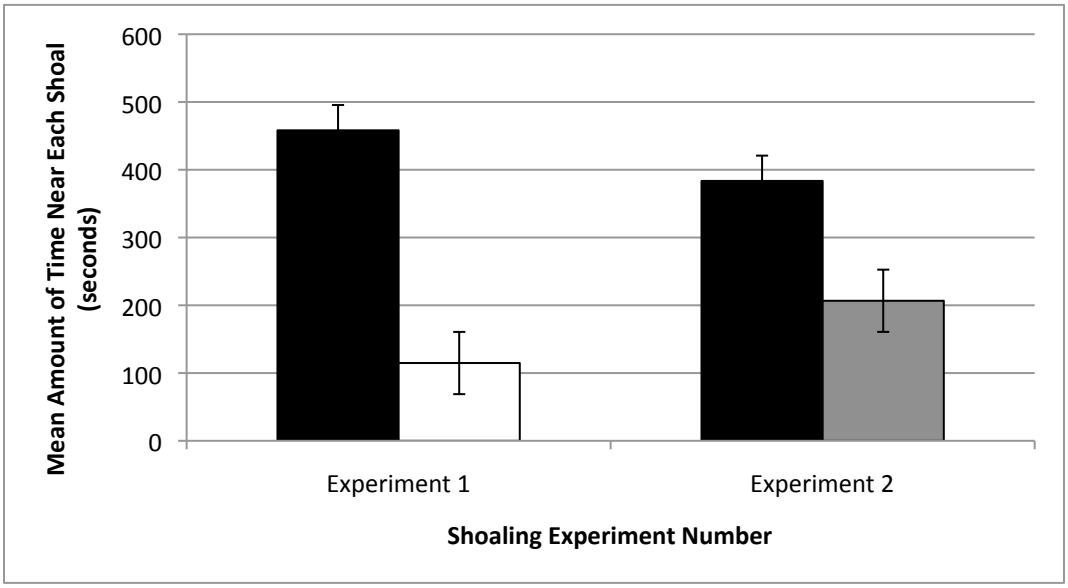

Figure3. Mean association time (+1 SE) in seconds of zebrafish individual's preference in the choice of (shoaling experiment 1): another shoal of zebrafish (black) and an empty compartment (white); or (shoaling experiment 2): a shoal of zebrafish (black) and a shoal of Glofish (gray). N= 15 for each shoaling experiment.

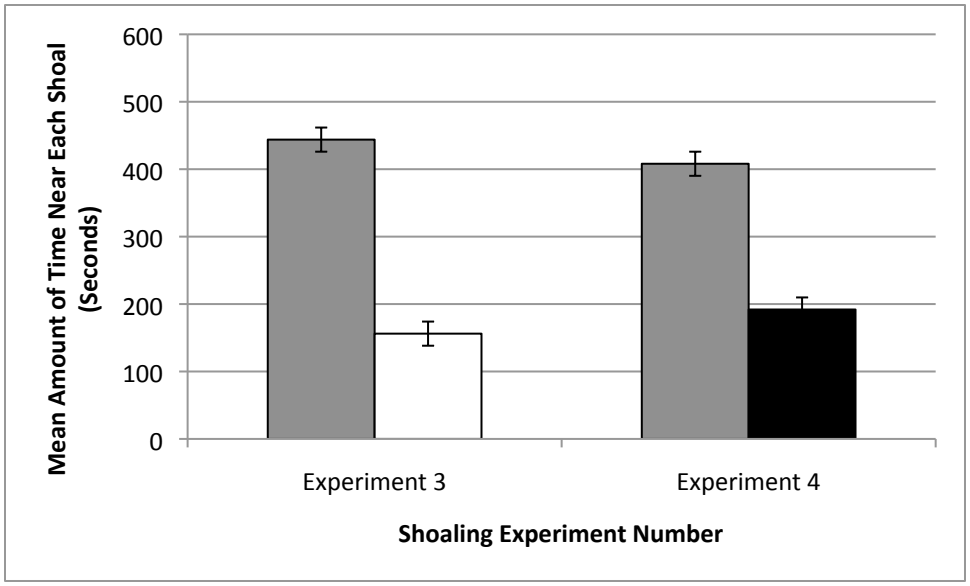

Figure 4. Mean association time (+1 SE) in seconds of Glofish Preference in the choice of (shoaling experiment 3): another shoal of Glofish (gray) and an empty compartment (white); or (shoaling experiment 4): a shoal of Glofish (gray) and a shoal of zebrafish (black). N=15 for each shoaling experiment.

Figure 3 and Figure 4 both show that the Glofish individuals and the zebrafish preferred to spend more time near the shoal that shared the same phenotype when given the choice between a shoal of similar phenotype, a shoal of a different phenotype , or an empty compartment.

Table 2 exhibits the mean times in seconds that each individual spent near each shoal along with each p-value. Each p-value from the unpaired t-test is represented for each test number. All four p-values in each separate test show a significant difference between the amount of time spent on each side compared to the null hypothesis of spending 300 seconds on each side.

Table 2. Each test number with mean associated times (seconds) and p-values is represented. N= 15 for each test.

Shoaling Experiment Number	Shoal 1	Mean Time (seconds)	Shoal 2	Mean time (seconds)	P-value
1	Zebrafish	458.2	Empty	114.8	<0.001
2	Zebrafish	393.53	Glofish	206.47	0.000152
3	Glofish	443.87	Empty	156.13	<0.001
4	Glofish	408.07	Zebrfish	191.93	0.000123

An ANOVA one way post hoc tukeys test was used to compare each of the shoaling experiments with each other. The t-test had shown that there were some significant differences, however, the ANOVA test indicated that the most significant differences were in the zebrafish preference. Therefore, there were significant differences in both shoaling experiments 3 and 4 for zebrafish preference, which means that zebrafish do discriminate against yellow Glofish when forming shoals.

Overall, both zebrafish and yellow Glofish individuals prefer to spend more time near a shoal that is phenotypically similar instead of a shoal that is phenotypically different, or an empty compartment. The p-values for each of the shoaling experiments

exhibit a significant difference between the amount of time spent on each side compared to the null hypothesis, which means that zebrafish do discriminate against potential shoal mates based on phenotype.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The new development of genetically modified fish allows for the opportunity for studying the effects of differences in body coloration in social groups. Many studies in the past have been completed to look at fish that are similar in appearance, but have coloration differences, and observe how color influences shoaling behavior. These studies have been done to observe how the differences might change the shoaling behavior in the fish.

Differences were shown in each of the four shoaling groups. Figures 3 and 4 exhibited that when given a choice, a zebrafish individual and a Glofish individual chose to shoal with a group of fish with the same phenotype. The figures also exhibited that when given a choice between a shoal that is phenotypically similar and a shoal that is phenotypically different, both zebrafish individuals and Glofish individuals chose to shoal with the phenoypically similar group of fish. Each of the four shoaling experiment groups was expected to have individual fish spend an equal amount of time on each side, which was 300 seconds on each side. An unpaired t-test was used to compare the amount of time spent on each side compared to the null hypothesis of 300 seconds spent on each

side for each of the four experiment groups. Table 2 exhibited all of the p-values from each of the tests. The first two shoaling experiments focused on zebrafish preference. The p-value of test 1 was <0.001, which meant that there was a significant difference between the amount of time an individual zebrafish spent on near another shoal of zebrafish and an empty container compared to the null expectation of spending an equal amount of time on each side, which would be 300 seconds.. The p-value of shoaling experiment group 2 was 0.000152. This p-value showed that there was a significant difference between the amount of time an individual zebrafish spent near another shoal of zebrafish and a shoal of Glofish compared to the null expectation of spending 300 seconds on each side of the tank.

The second two shoaling experiments focused on Glofish preference. The p-value of shoaling experiment group 3 was <0.001, which showed that there was a significant difference between the amount of time an individual Glofish spent near another shoal and to an empty container compared to the null expectation of spending an equal amount of time on each side. Shoaling experiment group 4 yielded a p-value of 0.000123. This p-value meant that there was a significant difference between the amount of time an individual Glofish spent near another shoal of Glofish and a zebrafish compared to the null hypothesis of 300 seconds spent on each side.

The data in the t-tests for all four shoaling group experiments yielded p-values that indicated significant differences. These p-values state that there are all differences in the amount of time an individual fish spent near the shoals compared to the null hypothesis. Since both zebrafish and Glofish prefer to stay near shoals that are phenotypically similar, it could mean that they both behave the same because they prefer

to be near phenotypically similar shoals. However, since both zebrafish and Glofish did not spend the same amount of time near each other, it shows that there may be discrimination against each other. This may mean that the zebrafish do not behave as though Glofish are the same. It may also mean Glofish also do not behave as though zebrafish are the same.

All of the four shoaling experiment groups yielded p-values of <0.001. These p-values meant that there was a significant difference between the amount of time spent on each side compared to the null hypothesis expectation of 300 seconds on each side. This significant difference between each of the experimental groups and the null expectation of 300 seconds does not support the hypothesis that both zebrafish and Glofish would spend an equal amount of time on each side. Therefore, for both zebrafish and Glofish, all individuals preferred to shoal with other fish as opposed to being alone. The t-test also shows that there may be discrimination between zebrafish and Glofish. However, a one way post hoc tukeys ANOVA test was conducted to observe if there were significant differences between each of the four shoaling experiments that would show discrimination. The results exhibited that there was in fact discrimination among the zebrafish and Glofish, but it was only in zebrafish preference. This meant that after observing the ANOVA results, there were only differences between the amount of time the zebrafish spend near the glofish. Therefore, zebrafish do discriminate against yellow Glofish, but yellow Glofish do not discriminate against zebrafish.

The original hypothesis stated that the individual fish would choose to shoal with phenotypically similar itself when it is given the choice between a shoal of phenotypically similar fish, a shoal of phenotypically different fish, and an empty

compartment. All four of the shoaling experiments showed that this hypothesis is supported, since individuals in all four shoaling experiments preferred to shoal with fish that were phenotypically similar.

Other studies such as one done by Snekser et. al. (2010) showed that all four strains of zebrafish used in the experiment preferred to shoal with fish more phenotypically similar to itself compared to shoaling with an empty compartment. The study done by Snekser et al. (2010) supports the data in this study that zebrafish and genetically modified zebrafish prefer to shoal with other shoals that look phenotypically similar compared to an empty compartment.

Another study done by Snekser et al. (2005) also proved that there was a significant difference between the amount of time zebrafish and genetically modified red Glofish spent with shoals that looked phenotypically similar and an empty compartment. Therefore, zebrafish and Glofish prefer to shoal with a phenotypically similar shoal instead of an empty compartment. The study done by Snesker et al. (2005) also supports the findings that both zebrfish and Glofish prefer to shoal with fish that are phenotypically similar. However the study done by Snesker et al. (2005) showed that both male and female fish of both zebrafish and red Glofish preferred to spend an equal amount of time with a shoal of three zebrafish, and a shoal of three red Glofish. The results found by Snesker et al. (2005), do not agree with the results found in this study. However, the Glofish used in this study were yellow and not red. Therefore, the coloration in the zebrafish seems to have a huge impact on the zebrafish's desire to shoal with the Glofish.

Another study done by Saverino and Gerlai (2008), also suggests that zebrafish do not discriminate against red transgenic Glofish. This study agrees with the previously mentioned study that zebrafish spend an equal amount of time among other zebrafish and red Glofish. However, both studies were conducted on red Glofish, while this study was conducted on yellow Glofish. It seems that zebrafish only discriminate against yellow Glofish, and they do not discriminate against red Glofish. Therefore, it seems very apparent that the zebrafish can discriminate against other potential shoalmates based on coloration. This suggests that genetically modifying an animal can affect its behavior and the behavior towards it of the animal it was modified from. This change of behavior seems to already by apparent in Glofish, which are the first genetically modified animals available to the public. Shoaling is an important behavior in a zebrafish individual, *Danio* rerio. Individuals use this behavior for protection from predators, selection of future shoalmates, and for efficient foraging. Discrimination from potential shoal mates, such as found in this study of zebrafish discriminating against Glofish, could keep the fish from obtaining the important benefits of shoaling. If further animals become genetically modified, it would be important to note any behavioral changes that could potential harm the animal.

A study conducted by Saverino and Gerlai (2008), noted that color has been found to be an important factor affecting choice behavior in fish. The study observed that red colored images of zebrafish, which are not naturally found in nature, were not avoided, the unaltered images of zebrafish were more significantly preferred (Saverino and Gerlai. 2008). However, zebrafish had a strong preference for yellow colored images. The reason for this was probably because vividly colored zebrafish exhibit more yellow coloration,

and therefore it is possible that the yellow colored computer image showed a trait that was interpreted by the zebrafish to indicate healthy or reproductively mature fish (Saverino and Gerlai 2008). However, this finding does not correspond with this study. This could possibly be because the Glofish used in this experiment were not completely yellow. They had a slight orange tint to them. The difference could also be in the fact that the yellow Glofish produce a much brighter glowing, than the yellow images. Another large difference between both studies could have been that the study conducted by Saverino and Gerlai (2008) was completed with computer images, while this study was conducted with real fish.

This study and other studies have shown that coloration does have an influence on shoaling decisions in zebrafish. So far, most studies have been done on red Glofish. However, Glofish have only been available to the public for a few years, and they have not been researched as much as zebrafish. Further studies would need to be done to look at all six of the current colors (red, yellow, green, orange, blue, purple) of zebrafish to see how the genetically modified Glofish's behaviors are similar or not similar to the behavior of zebrafish. It would be important to know if these colorations change either the Glofish's shoaling preference or the zebrafish's preferences to shoal with them. Knowing this could help understand how genetically modifying animals changes or doesn't change their behavior.

Genetic modification seems to be moving from plants to animals. Scientists have already seen positive and negative effects on plants, but changes in animal behavior could become a larger problem due to concerns about the animal's safety and environmental

safety. Understanding how genetically modifying animals influences their behavior is very important for modifying future animals.

APPENDICES

APPENDIX A

MARYVILLE COLLEGE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE
Application for Use of Vertebrate Animals in Student Research

Provide information after each bold item
Student Name: Kristen Barlow

Student Email Address: kristen.barlow@my.maryvillecollege.edu

Date: April 16, 2012

Senior Study Advisor: Jennifer Brigati

Species to be used: Glofish (Brachydanio rerio) and Zebrafish (Danio rerio)

Age of animals: approximately 6 months to 1 year old

Number of animals in study: 10 Zebrafish and 5 Glofish of one color and 5 Glofish of another color

Duration of study: Approximately a 2 month period

Location of animals during the study (building and room): Sutton room 114

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Kristen Barlow	865-556-6847	865-556-6847	865-556-6847
Jennifer Brigati	865-981-8168	865-318-6885	865-318-6885

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.
After the study, they will be kept alive and housed by the researcher, Kristen Barlow.

(Do not write below line: For MC IACUC Use)

Maryville College IACUC Approval Number: 2012-05
Date Approved: April 18, 2012
Signed:

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