INTRODUCTION

The zebrafish (*Danio rerio*), a small fish less than one inch in length and aptly named for its striped appearance, has revolutionized the fields of genetics and embryology in the last 30 years. These fish were first identified as a promising species for use in developmental biology and molecular genetics research in 1981 by Streisinger, Walker, Dower, Knauber, and Singer. However, they earned a permanent standing as a valuable model organism to genetics and embryology laboratories when, in 1996, the zebrafish became the subject of the first large-scale random mutagenesis screens to be done with a vertebrate, a process in which random mutations were generated and characterized in large numbers of fish, and the underlying genes were identified. This important project resulted in the identification of over 400 genes controlling vertebrate development (Granato & Nusslein-Volhard, 1996). Subsequently, a project began in 2001 to sequence the zebrafish genome (Sanger Institute, 2008), and since then there has been a flood of research utilizing the findings of the genome project to investigate the genetic basis of everything from the molecular pathways of drug abuse and addiction to molecular explanations of genetic disease in vertebrates (Fishman, 2001).

Zebrafish Ecology and Life Cycle

The zebrafish is a freshwater, shoaling fish species endemic to rivers, streams, and stagnant or slow-moving pools of water such as rice paddies in northeast India, Bangladesh, and Nepal (Engeszer, Patterson, Rao, & Parichy,

2007). Adult zebrafish are approximately one inch in length and reproduce via external fertilization. During mating, the female will release five to 20 eggs at a time while the male releases sperm, thereby fertilizing the eggs. A female can produce several hundred eggs in a single clutch. Zebrafish larvae hatch four to six days post fertilization and reach sexual maturity within four months. The normal lifespan of a zebrafish is three to five years (Spence, Gerlach, Lawrence, & Smith, 2008).

Zebrafish in Biological Research

The zebrafish is one of the most widely used vertebrate model organisms used in biological research today. Zebrafish first became popular in the fields of genetics and embryology because direct and complete observation of development is possible with relatively little time and expense. That is, fertilization and development occur outside of the female's body, the eggs are completely transparent, and the embryos themselves can be made transparent through a simple pigment mutation. Furthermore, development is rapid, with precursors to all major organs developing within 36 hours of fertilization. Additionally, zebrafish are hardy, they have a short generation time (typically three to four months), and a single pair can produce hundreds of eggs every few days. In many genetics and embryology laboratories, zebrafish compare favorably to other popular model organisms: zebrafish are more closely related to humans than invertebrate models such as *Drosophila melanogaster*, and although mice are more closely related to humans than zebrafish, experimental procedures

with zebrafish are often considerably less costly and complicated than similar procedures with mice (Spence et al., 2008).

Behavioral Assays

More recently, the scope of zebrafish research has grown to include the genetic underpinnings of behavior. Researchers have begun using behavioral differences between wild-type and mutant fish to assess the role of particular genes in the function of the nervous system. Well-designed behavioral assays are valuable because they are sensitive enough to detect subtle differences in nervous system development or functioning that could not be detected by histological screens. Additionally, behavioral assessment of gene function allows the researcher to see how mutation of a particular gene affects the functioning of a single neural system as it interacts with other neural systems in the brain, giving a more holistic view of the role of genes in the nervous system (Orger et al., 2004).

Behavioral assays for larvae. Many of the behavioral assays that have been developed to test mutations in the zebrafish nervous system are designed to test larvae. Using larvae rather than adult fish can be advantageous because larvae live for eight days after fertilization without needing food; moreover, because they are only a few millimeters in length, they require very little space. Thus, thousands of larvae can be housed in a laboratory while requiring relatively little maintenance (Orger et al., 2004). Furthermore, within just a few hours or days of fertilization, larvae display a number of behavioral patterns that are of interest to researchers, rendering the hassle and cost of raising the fish to

adulthood unnecessary. For example, Granato and Nusslein-Volhard (1996) investigated the genes underlying neuronal mediation and control of different patterns of motility in zebrafish larvae beginning at just 48 hours post fertilization. In their experiment, Granato and Nusslein-Volhard used a touch-response test to compare movement patterns between wild-type larvae and larvae with single gene mutations, thus revealing whether the gene was important to the fish's ability to execute a particular component of motility, for example, the ability to move their tail rhythmically from side to side during swimming.

Because of the simplicity of testing behavior in larvae and the short amount of time that researchers have been investigating behavior in zebrafish, there is a paucity of assays to test behavior in adult zebrafish. The assays that have been developed, however, show promise in their ability to assess the genetic bases of behavior more complex than those displayed by larvae, and thus there is a need for more behavioral assays that test adult fish (Spence et al., 2008).

Assays to identify the genetic basis of drug-addiction. Several studies have used adult zebrafish in behavioral assays to identify the genetic mutations that control an individual's predisposition to developing a drug addiction. Darland and Dowling (2001) used a conditioned place preference assay to identify zebrafish that displayed a decreased affinity for cocaine. To identify these individuals, the fish were placed in a two-chamber apparatus. Initial side preference was determined by the amount of time spent in each chamber during a two-minute period. After each fish's initial preference was established, it was

confined to the less-preferred side and exposed to cocaine. The following day, fish were returned to the apparatus and their preference was measured again. Fish that did not prefer the side on which they had been exposed to cocaine were identified and the underlying genes were found and sequenced. Researchers discovered that these fish differed from their cocaine-preferring counterparts by a single-gene mutation. In all likelihood, this mutation contributes to individual differences in predisposition to drug addiction in other vertebrate systems, including humans.

Gerlai, Lahav, Guo, and Rosenthal (2001) designed a series of simple behavioral tests to determine the effect that differing doses of alcohol have on a fish's locomotion, aggression, shoaling tendency, alarm response, light/dark preference, and pigmentation. Similar to the conditioned place preference assay in the study by Darland and Dowling (2001), these tests could be used to identify individuals that display abnormal responses to alcohol (e.g., heightened or decreased affinity). The genes underlying these abnormal responses to alcohol exposure could be subsequently identified and sequenced.

Another study using zebrafish behavior to investigate the genetic basis of drug addiction developed assays to measure a fish's startle response, as well as the degree of cohesion in a group of shoaling fish (Dlugos & Rabin, 2002). These measures were used to compare the effects of one-time exposure to alcohol (acute exposure) to continuous exposure over one or two weeks (chronic exposure) in three different strains of zebrafish. Fish from the three strains differed both in

their performances in the assays after alcohol exposure (acute or chronic) and in their level of tolerance to alcohol after chronic exposure. The results suggest a genetic basis of an individual's initial sensitivity and development of tolerance to alcohol, and that behavioral screening assays could lead to the identification and sequencing of the key genes involved (Dlugos & Rabin).

Assays to investigate the genetic basis of social behavior. Behavioral assays have also been used to determine genes that underlie social behavior. Larson, O'Malley, and Melloni (2006) observed pairs of male zebrafish for establishment of dominant-subordinate relationships over a period of five days. After dominance relationships had been established, researchers measured the expression of the neuropeptide arginine vasotocin in the brains of both dominant and subordinate fish. Arginine vasotocin is the teleost (bony) fish homolog of arginine vasopressin, a neuropeptide found in mammals whose expression has been linked to an individual's social position and level of aggression. The results suggest that the type, number, and location of brain cells in which arginine vasotocin is expressed differ significantly between dominant and subordinate fish. Therefore, changing dominance relationships result in differential expression of multiple genetic pathways. This finding illustrates how the central nervous system can mediate complex and constantly changing social behaviors.

Assays to determine patterns of behavioral deficits. Zebrafish behavior can be used to model patterns of behavioral deficits in humans. Tropepe and Sive (2003) have proposed that zebrafish could be useful in investigating the genes

underlying autism in humans. One approach to identifying genes of interest would be to use social behavior in zebrafish as a paradigm for sociability in humans. For example, mating behavior is a complex social behavior that is controlled by many genes. Mutated fish could be paired with a wild-type fish of the opposite sex and be observed for abnormal behavior. One could, for example, observe the level of interest displayed by a mutated male, or count the number of interactions initiated by a mutated male, and compare these behavioral patterns to behavior in wild-type males from the same family line. Gene mapping techniques could then lead to identification and sequencing of the genes that underlie decreased levels of interaction in courtship scenarios. If the behavioral patterns to be analyzed in zebrafish are selected carefully, it is likely that the genes underlying abnormal social interactions in fish would correspond to genes underlying abnormal social interactions in autistic humans.

In the past, researchers have modeled other types of behavioral deficits using tests of exploratory behavior in mice to measure anxiety and fear (Flint et al., 1995). Applying this concept to zebrafish, Wright, Nakamichi, Krause, and Butlin (2006) constructed a paradigm for anxiety and fear by measuring and comparing the willingness of wild versus laboratory strains of zebrafish to approach a foreign object, termed “boldness” in the nonhuman animal literature. Based on fishes’ performance in the assay, the researchers could determine boldness by examining the level of anxiety and fear experienced by the fish, with more anxiety and fear corresponding to less boldness. The results showed

significant differences in boldness between the wild and laboratory strains, and the researchers were able to identify particular loci underlying these phenotypic differences. Thus, Wright et al. discovered a genetic underpinning of an individual's position in a social hierarchy that is likely applicable to other vertebrates, including humans.

Assays to assess learning and memory function. Finally, several assays have been developed to quantify a fish's learning and memory abilities, and the effects that certain mutations, living conditions, or drug treatments have on learning and memory. Although these studies have not yet been utilized for discovering genetic underpinnings of behavior, they show great promise for future use in gene mapping experiments. For example, Levin and Chen (2004) demonstrated the usefulness of zebrafish as a model for studying the behavioral impact of neurotoxins in a study that investigated the impact of nicotine on learning and memory. Fish were placed in the center compartment of a three-chambered tank with partitions blocking access to the two side chambers. After a period of habituation, the partitions were raised, and when the fish entered a side chamber, the partitions were closed and a punishment ensued during which the partitions were moved along rails towards the ends of the tank to significantly reduce the size of the side chambers and impede movement of the fish. The side into which the fish first entered thus became the "incorrect" side, and any incorrect choices in subsequent trials resulted in the same punishment. After the first trial, the fish was returned to the center compartment and trained in eight

subsequent trials, during which the correct side alternated between the two side chambers. This procedure was repeated for 12 training days, after which the same fish were each tested in six different nicotine-exposure conditions varying by dosage.

Performance of the fish in the initial training period was compared to each fish's performance in the subsequent nicotine-exposure training sessions. The results showed that low doses of nicotine significantly improved memory performance, but that higher doses resulted in diminished performance on the task. This same trend has been observed in mammals (Levin and Simon, 1998), but the techniques involved in determining the molecular mechanism underlying this biphasic effect of nicotine on memory are easier to perform on zebrafish than on mammals. Thus, the discovery of a similar behavioral response between mammals and zebrafish to nicotine will aid in understanding how drug exposure can affect learning and memory in humans.

Another assay, developed by Bilotta, Risner, Davis, and Haggbloom (2005), investigated appetitive choice discrimination in zebrafish as a way to quantify learning ability. Fish were trained to use the presence of a light cue to swim into one of three chambers for a food reward. In the first phase of training, the fish was placed into the neutral area of a four-chambered tank. To create an association between the food reward and the light cue, the fish was presented with a food reward and the light cue upon entering any one of the three choice chambers. In the second phase of training, the light cue was presented in one of

the three choice chambers, and upon entering the lit chamber, the food reward was given. If the fish swam into one of the two incorrect choice chambers, the fish was confined to the chamber for 30 seconds and given no food reward. After three training sessions consisting of 20 trials each, the location of the correct chamber began to vary across trials. Fish were said to have learned the task when they made at least 80% correct choices in two consecutive training sessions. Each of 15 fish reached this criterion, though the number of sessions before reaching criterion ranged from six to 23. This study demonstrated that zebrafish are capable of learning a 3-alternative appetitive choice discrimination task, which allows experimenters to examine learning curves and retention for individual fish.

In a third study, Williams, White, and Messer (2002) developed a spatial alternation task in which fish were trained to swim to alternating sides of a divided tank for a food reward. A fish was said to have learned the task when it swam to the correct side upon presentation of a cue (tapping on the tank) in at least 75% of trials in a 28-trial session. The researchers showed that zebrafish mastered the task after just 14 trials, and could recall the task after 10 days without testing. In future research, this assay could be used to determine the phenotypic effects of a specific mutation, or to compare learning and memory abilities between wild and domestic fish, fish raised under different conditions, or fish with acute versus chronic exposure to drugs. Such versatility is inherent to most of the behavioral assays that have been developed for zebrafish, and thus

every behavioral assay developed creates innumerable new opportunities for future research.

Conditioned Place Preference

Another type of assay used for quantifying learning and memory ability is the conditioned place preference task (CPP). CPP is a type of associative learning in which the subject learns to associate a reward with a previously-neutral set of environmental cues. As the association is formed between the environmental cues and the location of reward, the subject will approach the cues more rapidly, more often, and/or will remain there for longer periods of time in anticipation of the reward, thus forming a measurable preference for the location of reward (Tzschentke, 2007). In nature, acquisition of CPP would be an adaptive mechanism. The ability to associate a set of environmental stimuli with the presence of food or another important resource, and thus be able to find that resource with minimal effort in the future, could increase the fitness of the animal. Because of this adaptive quality, most model organisms used in biological research, including mice, rats, fruit flies, and several species of fish, have the ability to acquire CPP (Tzschentke).

In zebrafish research, CPP has had two primary uses: to quantify learning and memory ability and to determine the motivational properties of drug or non-drug rewards (Yu, Tucci, Kishi, and Zhdanova, 2006). The latter is important because, as will be discussed later, the dysregulation of reward behavior can lead to prominent disorders such as obesity and addiction in humans (Lau, Bretaud,

Huang, Lin, & Guo, 2006). Quantifying learning and memory ability, however, is also important and lends itself to a wide range of applications. For example, as previously discussed, Levin et al. (2004) used CPP to determine the memory abilities of individual fish as a way to test the effects of nicotine on memory. In another study, Yu, Tucci, Kishi, and Zhdanova (2006) used CPP to test the effects of aging on cognition in zebrafish. Young (one-year-old), middle-aged (2-year-old), and old (3-year-old) zebrafish were fed daily on the red side of a tank that was half red and half white. After five days, young fish spent significantly more time on the red side of the tank in the 15 minutes preceding feeding than they had before training began. It took seven days of training for middle-aged fish to develop a significant preference for the red side preceding feeding, and old fish did not show any change in preference for the red side after seven days of training.

The same study (Yu et al., 2006) also used CPP to examine how environmental factors may interact with age to affect cognition in zebrafish, and tested a possible therapeutic intervention for preventing deterioration of cognition in old age. A group of young fish was exposed to gamma-irradiation before training, a process that in previous research has been shown to accelerate the effects of aging (Tsai et al., 2007). As expected, these gamma-irradiated fish resembled old fish in their performance on the CPP task, demonstrating that environmental factors can indeed alter the timing of cognitive effects of aging. As a potential therapy to the effects of aging, researchers tested zebrafish from all

three age groups that carried a mutation causing increased acetylcholine levels in the brain, a neurotransmitter found in previous research on human aging to be an important factor in cognitive aging (Bartus, Dean, Beer, & Lippa, 1982). Accordingly, as expected, the mutated zebrafish with modified cholinergic systems performed better than their wild-type counterparts, with both young and middle-aged mutated fish performing the same as young wild-type fish on the CPP task. Thus, gene therapies that increase acetylcholine expression may eventually be useful in preventing cognitive deterioration in aging humans.

CPP assays also have been developed to test the motivational properties of a drug. As previously discussed, CPP has been used to measure individual fishes' affinity for cocaine, which can be used as a screen for behavioral phenotypes of interest (Darland & Dowling, 2001). Braida et al. (2007) adapted the CPP paradigm developed by Darland and Dowling to investigate the rewarding properties of the recreational hallucinatory drug, salvinorin A. Their study revealed that when fish were injected with low doses of salvinorin A and then immediately confined to one chamber of a two-chambered tank, the fish developed a preference for the drug-associated side as demonstrated in subsequent tests by an increase in time spent in that chamber. In humans, too, salvinorin A carries significant rewarding properties, confirming zebrafish as a practical model organism for future research on the heretofore-unstudied addictive properties of the drug.

Finally, Lau et al. (2006) completed a study that identified as different the molecular pathways by which natural-reward behavior (food) and drug-reward behavior operate. One group of wild-type fish was trained to associate one of two chambers with morphine, while another wild-type group was trained to associate one of two chambers with a food reward. Both groups demonstrated a robust ability to acquire the CPP paradigm. Researchers then tested groups of fish carrying a mutation on both CPP paradigms. The mutation, called the *too few* mutation, is a single-gene mutation that causes fish to lack select dopaminergic and serotonergic neurons in a specific part of the brain, the basal diencephalon. These neurons had been shown in previous research to be involved in reward-associated behavior, though it was not known if they were involved in all types of reward-associated behavior or only drug-related behavior (Rink & Guo, 2004). The *too few* fish were no longer able to acquire the morphine CPP task but could still acquire the food CPP task normally. Similarly, wild-type fish that were pre-treated with dopamine receptor antagonists exhibited a normal food preference but lacked morphine preference, suggesting that both dopaminergic and serotonergic neurons are involved in drug-reward responses but not in natural-reward responses. Wild-type fish that were pre-treated with the opioid receptor antagonist naloxone were not able to aquire either task, suggesting that both drug-reward and food-reward behaviors involve the opioid system. Studies such as these suggest the possibility of characterizing the specific neuronal circuitries involved in different types of reward and may lead to therapeutic interventions

that curb drug-addictive behavior without affecting healthy, natural-reward-seeking behaviors.

Long-term Potentiation in the Central Nervous System

The most-widely accepted physiological model of learning and memory formation is long-term potentiation (LTP) (Malenka & Nicoll, 1999). LTP occurs when the pre-synaptic axon of one neuron and the post-synaptic dendrite of a neighboring neuron are activated simultaneously. As this synchronous activity is repeated, the post-synaptic neuron becomes increasingly sensitive to the neurotransmitter signals sent by that particular pre-synaptic neuron by increasing the number and activity level of particular neurotransmitter receptors on the post-synaptic cell surface. As a result, communication across the synapse is improved and the connection between those neurons is strengthened; that is, neurons that "fire together, wire together," to use the common phrase (Hebb, 1949). Thus, as an individual acquires new information, new pathways between neurons in the neural net are forged and strengthened (that is, potentiated), resulting in learning and the formation of memories (McNaughton & Morris, 1987).

The Role of GAP-43 in Long-term Potentiation

In 1986, Lovinger,Colley, Akers, Nelson, and Routtenberg found that one hour after LTP was induced in the hippocampus of adult rats, the activation of one protein in particular correlated highly with the location and magnitude of potentiation. This protein was later found to be the same as several other proteins already known to be involved in axon growth and LTP. These proteins, namely

protein F1, GAP-43, B_{50}, and pp_{46}, are now recognized as identical and are called GAP-43 (Gispen, De Graan, Chan, & Routtenberg, 1986; Nelson, Routtenberg, Hyman, & Pfenninger, 1985; Snipes, Chan, McGuire, Costello, Norden, Freeman, & Routtenberg, 1987).

GAP-43 (growth associated protein 43), a synaptic protein, is activated when another protein attaches a phosphate group to it, a process called phosphorylation. The primary protein to deliver the phosphate group--that is, to phosporylate and thereby activate GAP-43--is protein kinase C. When GAP-43 is unphosphorylated, it binds to the regulatory protein calmodulin, which stabilizes GAP-43 and prevents it from being phosphorylized in response to minor, transient signals. The presence of calcium ions detach calmodulin and allow for GAP-43 phosphorylation (Routtenberg, 1985).

When a synapse experiences repetitive activation and LTP is induced, a signaling cascade is triggered, resulting in the intracellular elevation of calcium ions and diacylglycerol, a signaling lipid. These changes activate protein kinase C and facilitate its translocation from the cytoplasm, or the interior of the cell, to the presynaptic membrane. Already in the presynaptic membrane are concentrated amounts of unphosphorylated, calmodulin-bound GAP-43. The increase in calcium ions causes GAP-43 to release the calmodulin and become phosphorylatable. The newly-arrived protein kinase C then phosphorylates the GAP-43 molecules and causes a sharp rise in the level of activated GAP-43 in the

membrane. Significantly elevated levels of activated GAP-43 can be observed within five minutes of the induction of LTP (Routtenberg, 1985).

Once phosphorylated, GAP-43 can have both long-term and short-term effects on synaptic efficacy. In the short term, increases in phosphorylated GAP-43 enhance neurotransmitter release by helping the synaptic vesicles, which are membrane-bound vesicles in which neurotransmitters are held, reach the presynaptic membrane, and by priming the presynaptic terminal to release more neurotransmitter if the signal is received again (Routtenberg, 1985). In the long term, increases in phosphorylated GAP-43 encourage outgrowth of nerve and presynaptic terminals. Phosphorylated GAP-43 has been shown to bind to filaments on the membrane skeleton, the network of proteins on the inner face of the membrane that directs its shape and motility. Upon binding to a skeleton filament, phosphorylated GAP-43 activates actin polymerization, the process by which the membrane skeleton changes shape (Meiri & Gordon-Weeks, 1990). Through this interaction, GAP-43 can influence the presynaptic membrane to cause larger and more numerous outgrowths, thereby remodeling and improving the efficacy of the synapse (Aigner et al., 1995). As evidence for GAP-43's role in encouraging neural outgrowth, Aigner et al. demonstrated that transgenic mice over-expressing GAP-43 show spontaneous nerve sprouting at the axon terminal. Over-expression also results in enlarged nerve endings of primary olfactory neurons in the olfactory bulb (Holtmaat et al., 1995).

During development, neurons rich in phosphorylated GAP-43 have the ability to extend axons to reach their synaptic targets. Once a neuron has formed mature synaptic connections, it loses the ability for axon growth and GAP-43 expression declines sharply. Interestingly, studies on humans and primates have shown that cortical regions associated with higher-order associations (Mishkin, 1982) continue to show elevated GAP-43 expression throughout adulthood (Nelson, Friedman, O'Neill, Lewis, & Routtenberg, 1987), while primary sensory and motor regions only express GAP-43 at very low levels. These findings support the idea that LTP, facilitated by GAP-43, allows specific neuronal populations to undergo structural changes related to information storage (Benowitz & Routtenberg, 1997).

GAP-43 in Nerve Development and Regeneration

The ability of GAP-43 to interact with the membrane skeleton allows GAP-43 to guide axon growth in multiple contexts. In LTP, GAP-43 encourages growth of the axon to reorganize and improve the synapse. In development, GAP-43 is instrumental to axon guidance during the formation of the neural net. The importance of GAP-43 to this process is illustrated by the work of Strittmatter, Fankhauser, Huang, Mashimo, and Fishman (1995), who found that mice bearing a mutation that disabled normal functioning of GAP-43 showed defects in axon pathfinding during development, defects that proved lethal shortly after birth.

In a similar capacity, GAP-43 is important to the regeneration of damaged neurons. After development is complete, some neurons have the ability to re-initiate neuron growth in response to axonal damage. This nerve regeneration occurs readily in the peripheral nerve systems of all animals, although only fish and amphibians are known to have the ability to initiate regenerative axon growth in the central nervous system. In both peripheral- and central-nerve regeneration, the GAP-43-dependent mechanism of axon elongation and guidance is essentially the same as during development (Benowitz & Routtenberg, 1997).

Effects of GAP-43 Gene Expression on Learning and Memory

The pivotal role of GAP-43 protein in learning and new memory formation has been demonstrated in numerous behavioral studies using rodents as a model organism (e.g., Rekart, Meiri, & Routtenberg, 2005; Routtenberg, Cantallops, Zaffuto, Serrano, & Namgung, 2000; Young, Owen, Meiri, & Wehner, 2000). For example, in the study by Rekart, Meiri, and Routtenberg, they demonstrated that GAP-43-deficient mice are significantly impaired in learning fear conditioning tasks. These mice were heterozygous for GAP-43 (symbolized as $GAP^{+/-}$) and thus showed a 50% reduction in GAP-43 expression from wild-type mice. Heterozygous mice were used because the homozygous mutation that completely eliminates GAP-43 expression ($GAP^{-/-}$) becomes lethal soon after birth. $GAP^{+/-}$ mice, conversely, develop normally and appear behaviorally normal on a gross level (Strittmatter, Fankhauser, Huang, Mashimo, & Fishman, 1995). The responses of $GAP^{+/-}$ mice were first observed during an

incremental series of foot shocks, which determined that the perceptual-motor capabilities of $GAP^{+/-}$ mice were similar to those of wild-type mice. Afterward, $GAP^{+/-}$ and wild-type mice were placed in a conditioning cage where they experienced a tone followed by a shock. Freezing behavior, indicative of fear level, was observed. $GAP^{+/-}$ and wild-type mice that were returned to the conditioning cage and presented with the tone one hour after training both showed comparable increases in freezing behavior upon hearing the tone. However, when placed in the conditioning cage 24 hours after training, the context in which they experienced the tone-shock pairing, $GAP^{+/-}$ mice showed less than one-half the amount of freezing behavior before presentation of the tone than their wild-type counterparts, although they did show a significant increase in freezing behavior from baseline levels. When presented with the tone, however, freezing behavior of the $GAP^{+/-}$ mice did not differ from the wild-type mice. These results suggest that a 50% reduction in GAP-43 expression inhibits but does not eliminate the ability to create contextual fear-related memories.

Conversely, the overexpression of GAP-43 in transgenic mice leads to significantly better performance on a maze task than wild-type mice (Routtenberg, Cantallops, Zaffuto, Serrano, & Namgung, 2000). Once each day for four consecutive days, mice were placed in the center of a maze with eight arms extending from the center start location. A food reward was available at the end of each of the eight arms. After the mice had retrieved four food rewards, they were removed for one minute, and upon returning to the maze, mice were

expected to use spatial memory to retrieve the remaining four rewards. In a second paradigm, only one arm of the maze was baited, which varied randomly on each of 23 days of training. Upon finding the food reward, mice were removed from the maze for one minute. During the delay, the maze was rotated and a new food reward was placed at the same spatial location as the first reward (though now in a different arm). Upon returning to the maze, mice were expected to use spatial memory to locate the food reward.

Three lines of transgenic mice were tested and their performance was compared to that of wild-type mice from the same family line. Mice in the first transgenic line overexpressed phosphorylatable (i.e., potentially activatable) GAP-43. Mice in the second transgenic line overexpressed unphosphorylatable (i.e., not activatable) GAP-43. Mice in the third transgenic line overexpressed permanently-phosphorylated (i.e., permanently activated) GAP-43. During the training periods of mice in all groups, electrodes implanted in the hippocampus delivered a small electric current to directly stimulate long-term potentiation. As expected, mice that overexpressed phosphorylatable GAP-43 performed significantly better on the spatial memory task than wild-type mice. Interestingly, mice that overexpressed permanently-phosphorylated GAP-43 also showed enhanced performance. Mice that overexpressed unphosphorylatable GAP-43 showed no better performance than wild-type mice. These results support the idea that phosphorylated, but not unphosphorylated, GAP-43 plays a direct and

important role in spatial learning and memory (Routtenberg, Cantallops, Zaffuto, Serrano, & Namgung, 2000).

Although the evidence from rodents for the role of GAP-43 in learning is compelling, one must bear in mind that GAP-43 transgenic rodents express abnormal amounts of GAP-43 throughout development as well as into adulthood (Routtenberg et al., 2000). Because GAP-43 is involved in the initial wiring of neural connections during development (Skene et al., 1986), the behavioral changes observed in adult rodents could be the result of abnormal wiring during development rather than the result of changes made to neural wiring in the adult (Routtenberg et al., 2000). Therefore, to confirm GAP-43's role in long-term potentiation, further experimentation with animals that develop with normal expression of GAP-43 is necessary.

Controlling Gene Expression with a Heat-Shock Promoter

Zebrafish provide a unique opportunity to study the effects of abnormal GAP-43 expression in adulthood while maintaining normal GAP-43 expression throughout development. In zebrafish, expression of an inserted GAP-43 gene can be controlled by a promoter, or region of DNA that controls whether a gene is "on" or "off," from another gene. This promoter would normally control activation of a gene encoding a heat-shock protein, a protein that is produced in response to stressors such as extreme heat (heat shock). Thus, when the heat-shock promoter is attached to the inserted GAP-43 gene, heat shock induces production of GAP-43 (Adam, Bartfai, Lele, Krone, & Orban, 2000). Use of the

heat-shock promoter to control the GAP-43 gene allows researchers to raise zebrafish to adulthood with normal GAP-43 expression, then induce abnormal expression of GAP-43 at any time during adulthood. Once a heat-shock-controlled mutation is induced, the effects of the mutation last approximately 24 hours, after which the fish return to wild-type expression of all genes (A. Udvadia, personal communication, April 1, 2009). By using this heat-shock-controlled mutation, one can rule out the possibility that behavioral differences between wild-type and transgenic fish are caused by GAP-43-related developmental abnormalities.

Current Study

My project consisted of two main goals, the first of which was to design a behavioral assay that could quantify the learning ability of adult zebrafish. To accomplish this goal, I developed a conditioned place preference assay that measured each fish's ability to learn a spatial task. The assay I developed is an improvement upon existing assays because previously developed experiments using conditioned place preference to test zebrafish have moved fish from home tanks to experimental tanks before training every day, a stressful procedure that involves netting the fish and moving them to new water. Consequently, procedures for these assays often must include several hours of habituation to the new environment before training or testing can begin, a procedure that significantly increases the time required to train or test fish each day. The assay I developed used a modified tank design that allows fish to live in the experimental

tank for the entire duration of the experiment, significantly reducing stress for the fish and decreasing the amount of time required per day to train the fish.

Additionally, conditioned place preference protocols often expose fish to the treatment or reward only once. Therefore, the only result generated by these experiments is whether or not fish learned an association during a single trial. I trained fish for five consecutive days, a procedure that can reveal the learning curve of individual fish. Furthermore, because most previously-existing assays have only one trial, the tank is often designed so that fish can swim freely between two compartments. With that design, the only way to initiate a new trial would be to remove the fish from the experimental tank and immediately return it. The tanks I used have movable partitions barring access to the side chambers, allowing the experimenter to run the fish through multiple consecutive trials without removing the fish from the tank. Finally, in a pilot study, approximately 70% of wild-type fish tested were able to learn the assay I designed. Thus, this assay could be used in studies in which significant improvement is expected, as well as in studies in which significant worsening of learning abilities is expected.

The second goal of my project was to use the conditioned place preference assay to compare the learning abilities of wild-type zebrafish to those of transgenic zebrafish that over-expressed the GAP-43 protein after heat shock. I tested the performance of three lines of transgenic fish in the conditioned place preference assay, as well as a group of wild-type fish (WT) that were from the same genetic line as the transgenic fish. The first transgenic line overexpressed

the wild-type GAP-43 gene (GAP-43^{+}). I expected a higher percentage of GAP-43^{+} fish learn to the task and/or to master the task more rapidly in training compared to WT fish, results that would support the hypothesis that GAP-43 facilitates learning and memory in zebrafish.

The second line of transgenic fish overexpressed an unphosphorylatable (i.e., unable to be activated) version of GAP-43 after heat shock (GAP-43^{-}). GAP-43^{-} was a control group that overexpressed the protein of interest but knocked out the function of that protein. Thus, this group determined the effects of simply having more GAP-43 in the cell, as opposed to having more activated GAP-43 in the cell. GAP-43^{-} was expected to show one of two patterns of behavior: GAP-43^{-} fish could show a similar performance in learning to WT fish, which would suggest that the unphosphorylated form of GAP-43 is non-functional but does not interfere with the functioning of the endogenous (and phosphorylatable) GAP-43 protein. Alternatively, the GAP-43^{-} fish could perform worse on the learning task than the WT fish, thereby showing a diminished capacity for learning and memory. A worse performance would suggest that the unphosphorylatable form of GAP-43 is non-functional, but because it was expressed at very high levels in the cell, the mutant GAP-43 competed with endogenous GAP-43 for binding to protein kinase C, the protein that activates GAP-43, as well as binding sites on the presynaptic membrane. Thus, high levels of unphosphorylatable GAP-43 could effectively cause an

under-expression of functional GAP-43 in nerve cells (A. Udvadia, personal communication, September 16, 2008).

The third line of transgenic fish expressed a membrane-bound red fluorescent protein after heat shock (WT^{red}). Because GAP-43 is a membrane-bound protein, WT^{red} was included as a control group to determine the effects on learning of over-expressing a membrane-bound fluorescent protein. I expected fish from WT^{red} to display learning abilities similar to WT fish; such results would confirm that higher levels of membrane-targeted protein in nerve cells has no effect on learning. All three lines of transgenic fish also expressed a nuclear-targeted green fluorescent protein, which was used as a marker in subsequent brain analysis to confirm the location and extent of transgene expression in the brain.

METHODS

Subjects

Zebrafish were bred and raised into adulthood in the genetics laboratory of Dr. Ava Udvadia at the Wisconsin Aquatic Technology and Environmental Research Institute. By breeding and engineering the mutant fish in her own lab, Dr. Udvadia was able to ensure that all fish came from the same genetic background and carried the appropriate mutations. Upon reaching adulthood, groups of 60 fish were shipped to my laboratory for behavioral testing. As soon as they arrived at Mount Holyoke College, the zebrafish were housed in ten-gallon aquariums in mixed-sex groups for at least seven days before being used in the study. During this time, the fish were kept under a 14:10 light:dark light cycle at a water temperature of 25° C and were fed a diet of flake food (OmegaSea, Limited, Sitka, AK) and frozen brine shrimp (Hikari, Incorporated, Hayward, CA) once daily. The fish also were closely observed for signs of illness or injury, and unhealthy fish were excluded from the study.

In total, there were 3 replications of 12 fish each. Within each replication, 3 fish were tested from each of the four groups (GAP-43^{+}, GAP-43^{-}, WT^{red}, and WT). Half of the fish from each group were heat shocked and the other half were not. After use in the study, all fish were sacrificed by exposing them to a highly concentrated aqueous solution of tricaine methanesulfonate. The brains of sacrificed fish were sent to Dr. Udvadia's laboratory, where they were analyzed to ensure that all fish were expressing the appropriate mutations.

Apparatus

The test fish were individually housed in the center compartment of a three-chamber test tank, pictured in Figure 1, for the length of the experiment. These tanks were also kept on a 14:10 light:dark light cycle with a water temperature of 25°C. The two side chambers were blocked by partitions that could be raised by experimenters standing approximately eight feet away from the test tank. The side chambers were decorated to help fish distinguish between the two sides: one chamber contained blue gravel, a tall red plant, and a square blue card on the wall. The opposite chamber contained purple gravel, a short green plant, and a square red card on the wall. The center chamber did not contain any gravel, plants, or cards on the wall. The back and side walls of the tank were covered in black, opaque plastic to minimize distractions and stressors visible to the fish during the experiment.

Procedure

The conditioned place preference task consisted of an initial preference test (Day 0), five consecutive days of training (Days 1-5), and a final preference test (Day 6). On Day 0, immediately before the initial preference test, fish were given a five minute habituation period during which both partitions were raised and fish were allowed to swim freely among all three chambers. The habituation period allowed the fish to explore the novel side chambers, which is important for preference formation. After the habituation period, fish were tested for their initial chamber preference. Again, both partitions were raised and the fish were

Figure 1. Picture of a three-chambered experimental tank.

allowed to swim freely among all three chambers for five minutes. During this test, the chamber in which the fish was located was recorded every 10 seconds for a total of 30 location observations. If the fish was in one side chamber for a greater number of observations than in the other, the fish was said to have an initial preference for that side. The chamber that the fish did not prefer initially became the "correct" chamber during training, namely the place in which a food reward was delivered to the fish during the training period. The opposite chamber, for which the fish showed an initial preference, became the "incorrect" chamber during training. For example, if a fish was in the left chamber for 18 observations in the initial preference test, the fish would be said to have an initial preference for the left chamber. Therefore, during training, the fish would be offered a food reward in the right chamber exclusively. If the fish learned the task successfully, it would then show a preference for the right chamber in the final preference test. For fish that did not leave the center chamber at all during the initial preference test or that spent equal amounts of time in each side chamber, the "correct" side was determined by a coin flip.

On Days 1 through 5, fish were trained to associate one side chamber with a brine shrimp food reward and the opposite side chamber with the absence of a food reward, a procedure loosely adapted from Bilotta, Risner, Davis, & Haggbloom (2005). Fish were not fed for one day prior to training to ensure motivation to perform the task. As zebrafish are capable of remaining healthy for

up to seven days without food, withholding food for one day did not compromise the health or behavior of the fish.

In each trial, partitions leading to the side compartments were raised approximately four inches. Fish then were given a choice period of two minutes in which they could enter one of the side chambers. Upon entering a chamber, the partitions were closed, confining the fish to the chamber. If the fish entered the correct chamber, it was given a small piece of brine shrimp in that chamber within 10 seconds; if it entered the incorrect chamber, it was not given any shrimp. One trial consisted of a fish entering one side chamber, staying for 30 seconds, and then returning to the center chamber. After the fish was in the center for 30 seconds, the partitions were raised and the next trial began. If a fish made no choice in the two-minute choice period, both partitions were closed and the fish was confined to the center for one minute, after which the partitions were raised to initiate the next trial. Each training session consisted of 10 consecutive trials; one training session was conducted per day. Thus, each fish received 50 trials over a period of five days. For each trial, the experimenter recorded the side the fish entered and the latency to enter that side.

Immediately preceding training sessions on Days 1 and 2, approximately 2 ml of water that smelled strongly of brine shrimp was added to each side chamber of the test tank. Adding shrimp-smelling water to the side chambers (called "chumming") encouraged the fish to venture out of the center chamber during the first two training sessions. Chumming was an important step: if the fish did not

leave the center chamber during training, they would not have the opportunity to discover the food reward and therefore could not have learned the task. For this reason, fish that make fewer than two choices during the first training session despite chumming, and fish that had not made any correct choices by the end of the second training session, were excluded from the study. Beginning on Day 3, the side chambers were no longer chummed.

On Day 6, after training was complete, fish were given a final preference test to determine whether the fish's chamber preference had changed. The final preference test was identical to the initial preference test; that is, both partitions were raised and the fish was allowed to swim freely between all three chambers for 5 min. The chamber in which the fish was located was recorded every 10 seconds for a total of 30 location observations. If the fish was in one side chamber for a greater number of observations than in the other, the fish was said to have a final preference for that side. There was no habituation period preceding the final preference test.

Heat Shock Protocol

On all seven days of the experiment, fish were exposed to either a heat-shock procedure or a heat-shock control procedure. Half of the fish from each of the four groups was exposed to the heat-shock procedure on all seven days, while the other half of the fish was exposed to the heat-shock control procedure on all seven days. For the heat-shock procedure, fish were removed from the experimental tanks and placed in individual 600-ml beakers, which contained 400

ml of water from each fish's experimental tank. The beakers were covered in opaque tape so the fish could not see out of them. Beakers were then covered in saran wrap and placed into a hot water bath, i.e., a larger tank containing 37°C water approximately 4 inches deep. The beakers had a layer of gravel on the bottom to prevent them from floating and/or tipping over. Water in the beakers reached 37° in approximately 45 minutes, after which point the beakers remained in the hot water bath for an additional two hours. After a total of two hours and 45 minutes in the hot water bath, beakers were moved to a cool water bath containing 25° water approximately 4 inches deep. Water in the beakers cooled to 25° in approximately 45 minutes. After cooling, fish were returned to their original experimental tanks.

The heat-shock control procedure was identical to that of the heat-shock procedure with the exception that beakers were placed in a 25° cool water bath instead of a 37° hot water bath. Thus, transgenic fish exposed to the heat-shock control procedure failed to have their mutations induced and were expected to have phenotypes no different than wild-type fish. By only exposing half of the fish to heat shock, I was able to determine the effect that the heat-shock procedure had on the fish's performance in the task.

RESULTS

Overview of analysis

Each of four groups of fish was further divided into two treatment sub-groups, namely "heat shock" and "no heat shock". Thus, there were eight groups for comparison. Each group of heat-shocked fish was compared to not-heat-shocked fish from the same group and, in a separate analysis, heat-shocked fish from the other three groups. The latter analysis was performed to reduce variation in the data set; because heat-shocked fish were exposed to a considerable amount of stress to which not-heat-shocked fish were not exposed, removing the not-heat-shocked fish from the analysis reduced variation among groups. Analyses were performed on data collected during the five training days as well as during the initial and final preference tests. The sample sizes of groups and sub-groups are listed in Table 1.

One criterion for inclusion in the study required fish to make at least one correct choice by the end of the second day of training. Thus, some fish made up to 10 correct choices on Day 1, thereby performing well, while others made no correct choices until, at the latest, the last trial on Day 2. This led to a high amount of variability in the percent of correct choices on the first and second days of training that did not accurately reflect learning ability. To correct for these potentially misleading differences in performance, two variables were created: the percent of correct choices on Days 1 and 2 combined, which diluted poor

Table 1

Sample Sizes of Groups and Sub-groups

	GAP-43^{+}	GAP-43^{-}	WT^{red}	WT	Total
Heat shock	5	4	3	6	18
No heat shock	6	3	3	6	18
Total	11	7	6	12	36

performances on Day 1, and the percent of correct choices on Day 2 alone, which eliminated poor performances on Day 1 altogether.

Another potentially misleading variable was the percent of time fish spent in the correct chamber during the final preference test. During training, fish received a food reward within 10 seconds of entering the correct chamber. In the final preference test, however, no reward was delivered upon entering the chamber; thus, after approximately 10 seconds the behavior could be extinguished. That is, a fish that learned the task well could enter the correct chamber, wait 10 seconds, and then spend the rest of the five-minute test in the center or incorrect chamber. This fish would receive a low score for the percent of time spent in the correct chamber during the test, but the score would not reflect the fish's learning of the assay. To correct for this discrepancy, a new variable was created, namely the percent of time spent in the correct chamber during the first 60 seconds of the test. However, some fish did not make any choices during the first 60 seconds but would choose the correct chamber later in the test. Thus, if only analyzing the first 60 seconds of the test, these fish would receive low scores when in fact they performed well. To correct for this discrepancy, the percent of time that a fish spent in the correct chamber in the 60 seconds immediately following its first choice was also analyzed.

In each of the following analyses, heat-shocked GAP-43^{+} fish were expected to outperform not-heat-shocked GAP-43^{+} fish and both heat-shocked and not-heat-shocked fish from the other three groups. In the GAP-43^{-}, WT^{red},

and WT groups, heat-shocked fish were expected to perform identical to not-heat-shocked fish. Furthermore, heat-shocked GAP-43^{+} fish were expected to perform better than heat-shocked fish from the GAP-43^{-}, WT^{red}, and WT groups.

The Percent of Correct Choices on Training Days

A mixed design Analysis of Variance (ANOVA) was used to compare the between-subjects variables, day and group, and the within-subjects variable, heat-shock treatment, for the percent of correct choices on each of five training days. The ANOVA revealed a significant three-way interaction between day, group, and heat-shock treatment for the percent of correct choices on each of the five days of training, $F(12,112) = 1.99$, $p = .032$. These data are illustrated in Figure 2. A t-test revealed that on Day 5, heat-shocked WT fish made a significantly lower percentage of correct choices (M = 34.29) than not-heat-shocked WT fish (M = 72.00), $t(9.21) = 2.60$, $p = .028$. The same pattern approached significance on Day 2, $t(10) = 2.00$, $p = .056$. Also approaching significance was the difference between heat-shocked GAP-43^{-} fish and not-heat-shocked GAP-43^{-} fish on Day 1, with the heat-shocked fish making a higher percentage of correct choices (M = 60.00) than not-heat-shocked fish (M = 3.33), $t(3.19) = -2.98$, $p = .054$. However, a between-groups design ANOVA revealed no significant

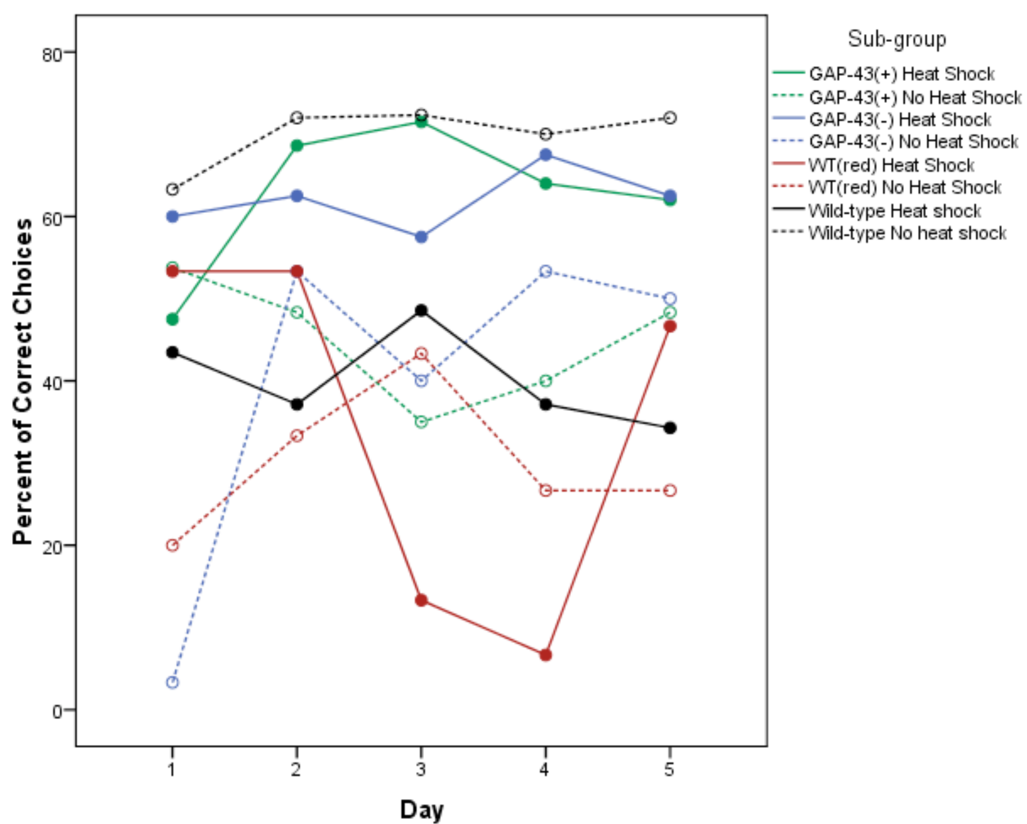

Figure 2. The percent of correct choices on each of five training days.

interaction between group and heat-shock treatment in the percent of correct choices on Days 1 and 2 combined, $F(3, 28) = 1.73$, $p = .184$, or on Day 2 alone, $F(3, 28) = 1.90$, $p = .152$. There were no significant main effects of group or heat-shock treatment (all $p > .05$). These data are illustrated in Figures 3 and 4, respectively.

A mixed design ANOVA revealed no significant differences among the four groups of heat-shocked fish in the percent of correct choices in any of the five days of training, $F(12, 56) = 1.76$, $p = .078$, and no significant main effects or two-way interactions (all $p > .05$). The comparison of heat-shocked fish over five training days is illustrated in Figure 5. There were also no significant differences among groups of heat-shocked fish on Days 1 and 2 combined, $F(3,14) = 0.51$, $p = .685$, as is illustrated in Figure 6, or on Day 2 alone, $F(3,14) = 1.22$, $p = .338$, as is illustrated in Figure 7.

The Number of Trials before the First Correct Choice on Training Days

A mixed design ANOVA revealed a significant three-way interaction between day, group, and heat-shock treatment for the number of trials that passed before the first correct choice was made in each of five days of training, $F(12,112) = 2.87$, $p = .002$. No significant main effects or two-way interactions were found (all $p > .05$). Figure 8 shows the number of trials that passed before the first choice was made for each sub-group over five training days. Investigating this three-way interaction further, a mixed design ANOVA

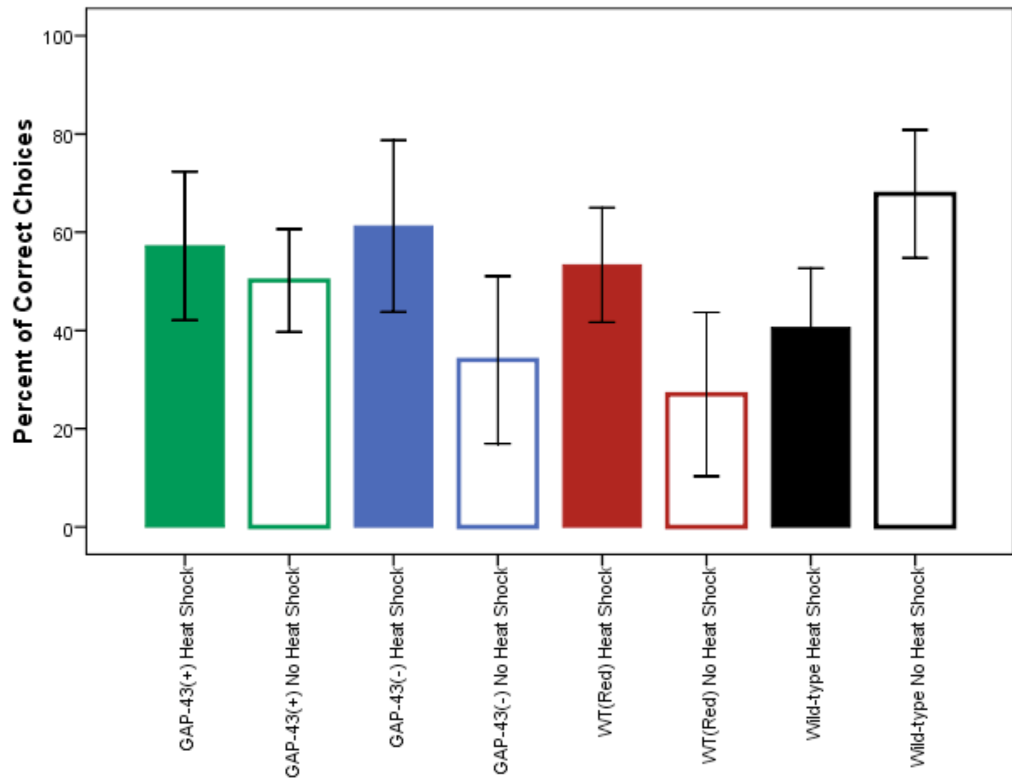

Figure 3. The percent of correct choices on Days 1 and 2 combined.

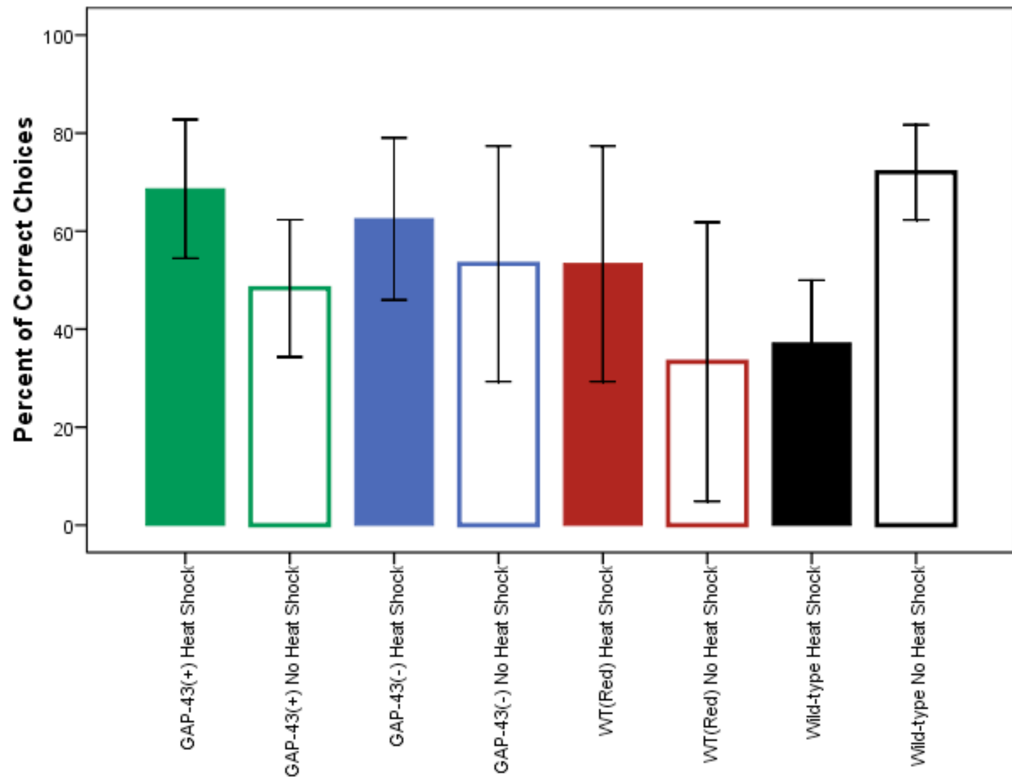

Figure 4. The percent of correct choices on Day 2.

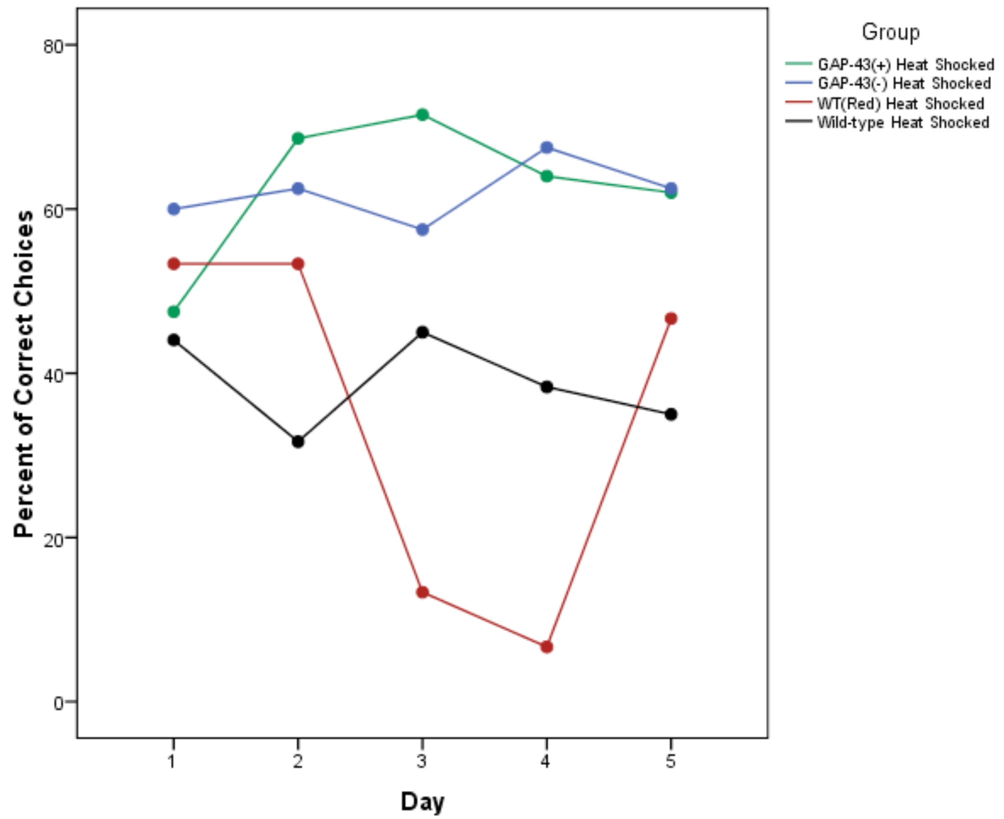

Figure 5. Comparison of heat-shocked fish on the percent of correct choices over five training days.

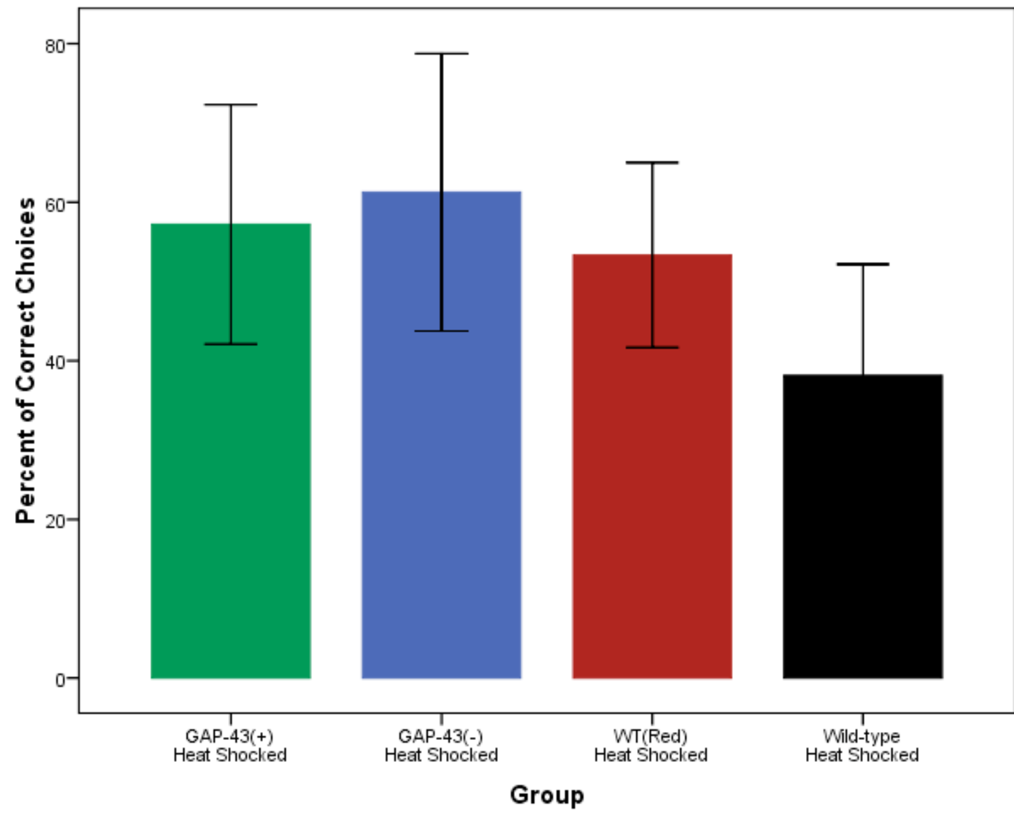

Figure 6. Comparison of heat-shocked fish on the percent of correct choices on Days 1 and 2 combined.

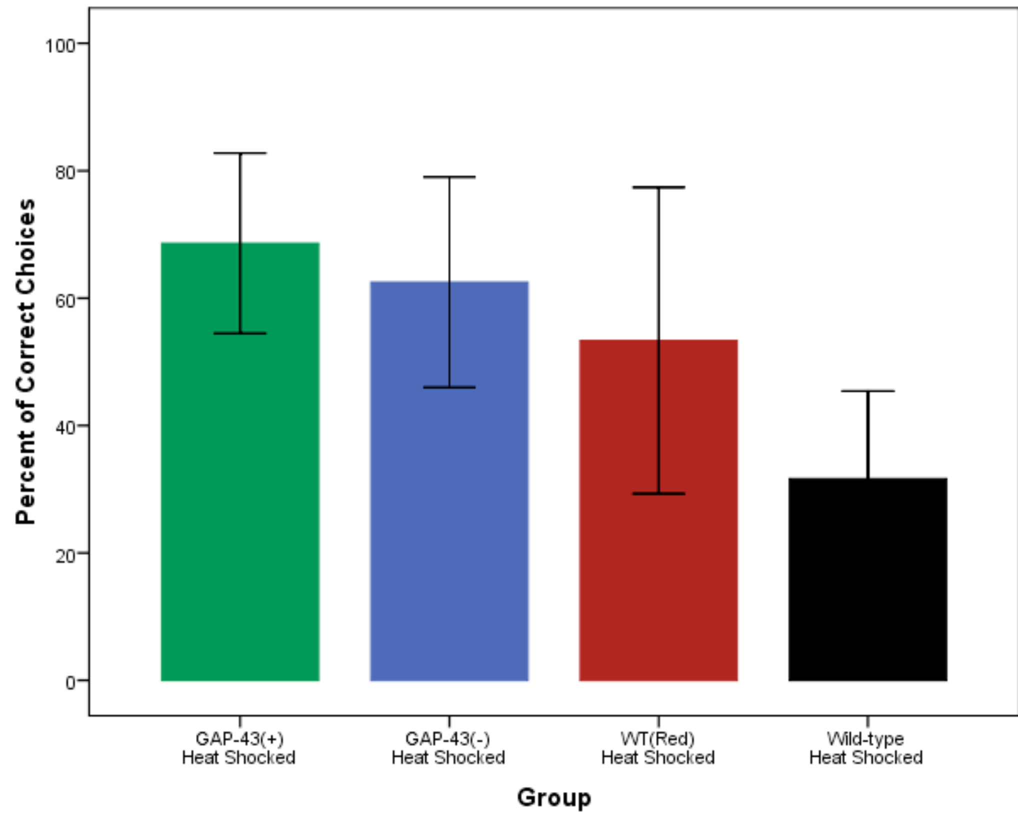

Figure 7. Comparison of heat-shocked fish on the percent of correct choices on Day 2.

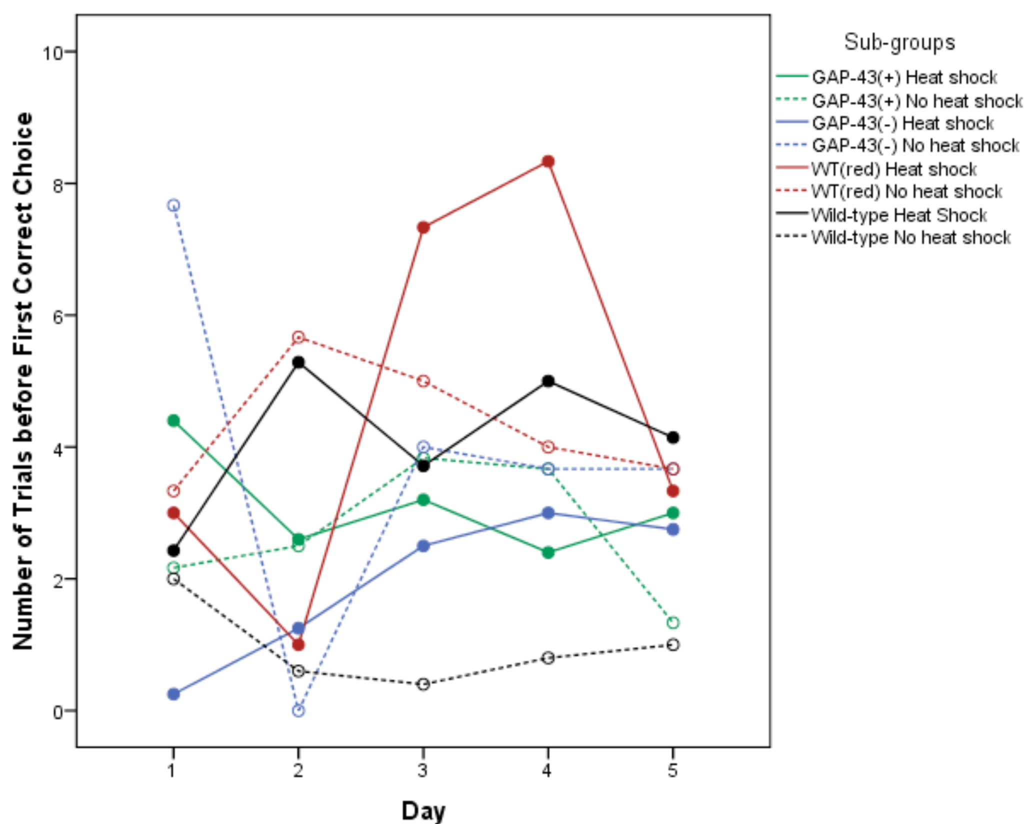

Figure 8. The number of trials that passed each training day before the first correct choice was made.

comparing heat-shocked and not-heat-shocked GAP-43^+ fish revealed no significant two-way interaction and no significant main effects (all *p*-values > .05), as is illustrated in Figure 9. However, a mixed design ANOVA did reveal a significant two-way interaction between day and heat-shock treatment for the number of trials that passed before GAP-43^- fish made their first correct choice, $F(4, 20) = 3.69$, $p = .021$. A *t*-test revealed that on Day 1, heat-shocked GAP-43^- fish waited for significantly fewer trials (M = 0.25) than not-heat-shocked fish from the same group (M = 7.67), $t(5) = 3.76$, $p = .013$. There were no significant differences between heat-shocked and not-heat-shocked GAP-43^- fish on any other days, and there were no significant main effects (all $p > .05$). These data are shown in Figure 10.

A mixed design ANOVA also revealed a significant two-way interaction between day and heat-shock treatment for the number of trials that passed before WT^{red} fish made a correct choice, $F(4, 16) = 3.53$, $p = .030$, illustrated in Figure 11. *T*-tests revealed that heat-shocked WT^{red} fish waited for significantly more trials before making a correct choice on Day 4 (M = 8.33) than they did on Day 2 (M = 1.00), $t(2) = -5.05$, $p = .037$ or Day 5 (M = 3.33), $t(2) = 8.66$, $p = .013$. Heat-shocked WT^{red} fish also waited for more trials before making a correct choice on Day 3 (M = 7.33) than on Day 2 (M = 1.00), and the difference approached significance, $t(2) = -3.59$, $p = .070$. No main effects were significant (all $p > .05$). There were no significant differences across days in the number of

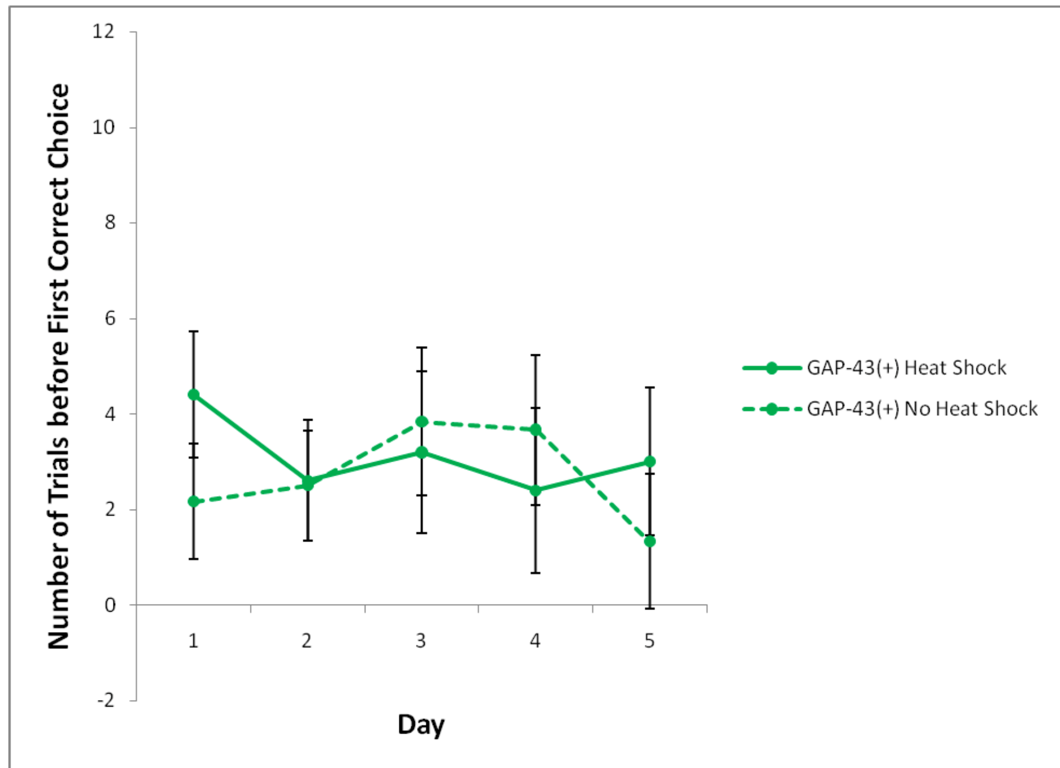

Figure 9. Comparison of GAP-43^{+} heat-shocked and not-heat-shocked fish on the number of trials that passed each training day before the first correct choice was made.

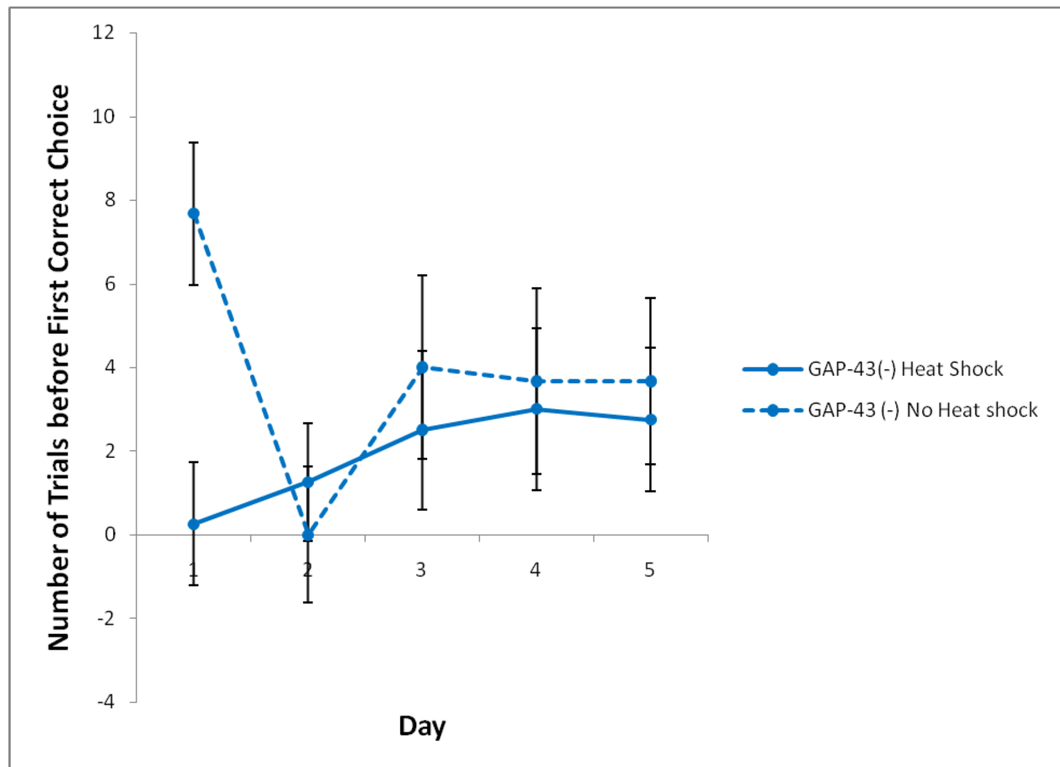

Figure 10. Comparison of GAP-43⁻ heat-shocked and not-heat-shocked fish on the number of trials that passed each training day before the first correct choice was made.

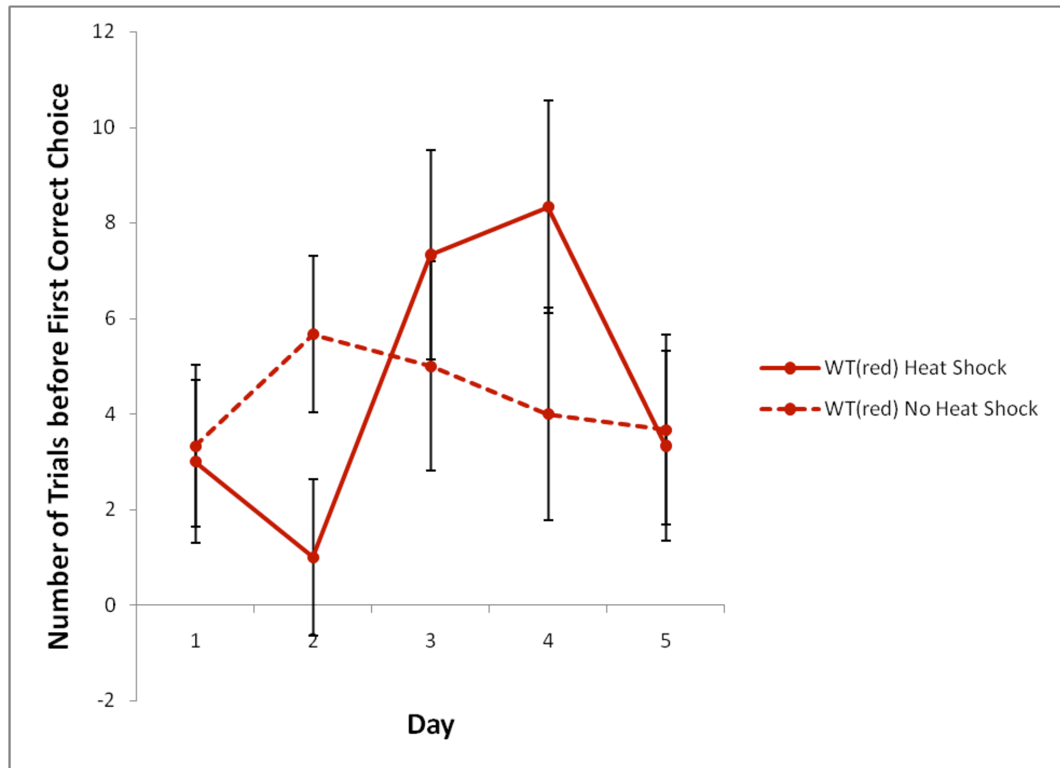

Figure 11. Comparison of WT^{red} heat-shocked and not-heat-shocked fish on the number of trials that passed each training day before the first correct choice was made.

trials that not-heat-shocked WT^{red} fish waited before making a correct choice, and no main effects were significant (all $p > .05$).

Finally, there was a significant two-way interaction between group and heat-shock treatment for the number of trials that passed before WT fish made their first choice, $F(4, 40) = 3.42$, $p = .017$, illustrated in Figure 12. A *t*-test revealed that on Day 2, heat-shocked WT fish waited for significantly more trials (M = 6.00) before making a correct choice than not-heat-shocked WT fish (M = 0.67), $t(10) = -3.67$, $p = .004$. The same trend approached significance on Day 3, $t(10) = -2.12$, $p = .060$. There were no significant differences between heat-shocked and not-heat-shocked WT fish on any other training days (all $p > .05$). The difference between heat-shocked and not-heat-shocked WT fish, regardless of day, approached significance with heat-shocked fish waiting for more trials (M = 4.33) before making a choice than not-heat-shocked fish (M = 1.27), $F(1, 10) = 4.55$, $p = .059$. Day was not a significant main effect, $F(4, 40) = 0.91$, $p = .467$.

A mixed design ANOVA revealed a significant interaction between day and the four groups of heat-shocked fish for the number of trials that passed before the first correct choice, $F(4, 56) = 2.57$, $p = .048$. These data are illustrated in Figure 13. A *t*-test revealed that on Day 1, heat-shocked GAP-43^+ fish (M = 4.40) waited for significantly more trials before making a correct choice than heat-shocked GAP-43^- fish (M = 0.25), $t(4.24) = 2.85$, $p = .043$. Furthermore, a *t*-test revealed that on Day 2, WT fish waited for significantly more trials

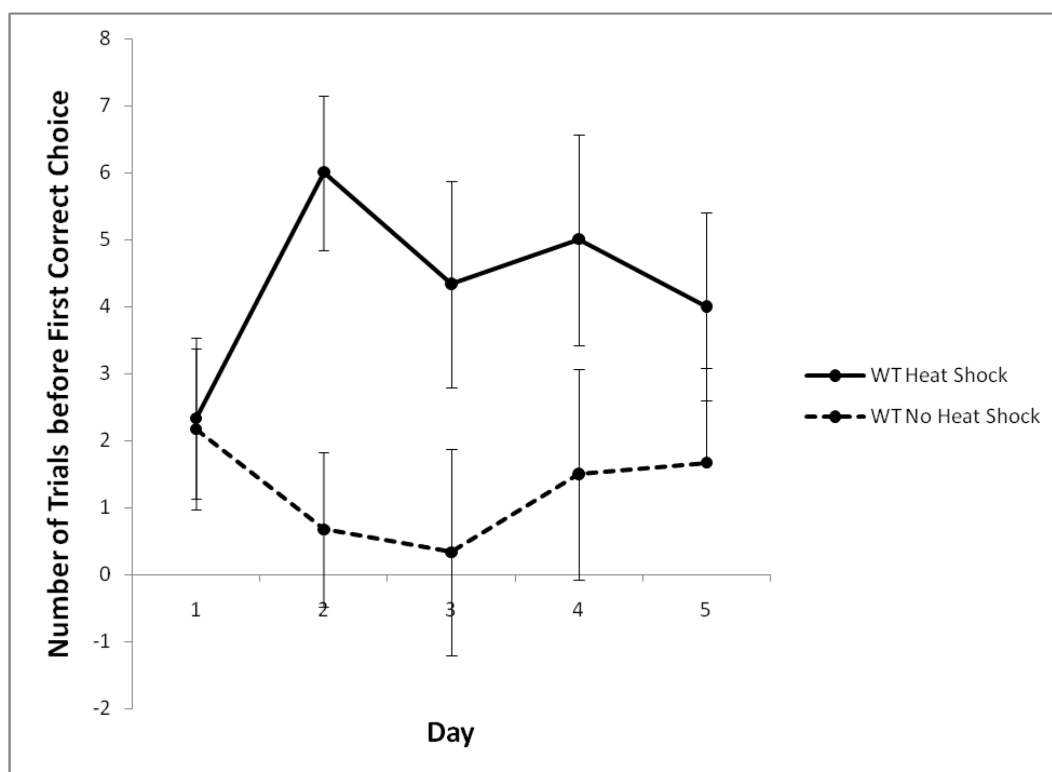

Figure 12. Comparison of WT heat-shocked and not-heat-shocked fish on the number of trials that passed each training day before the first correct choice was made.

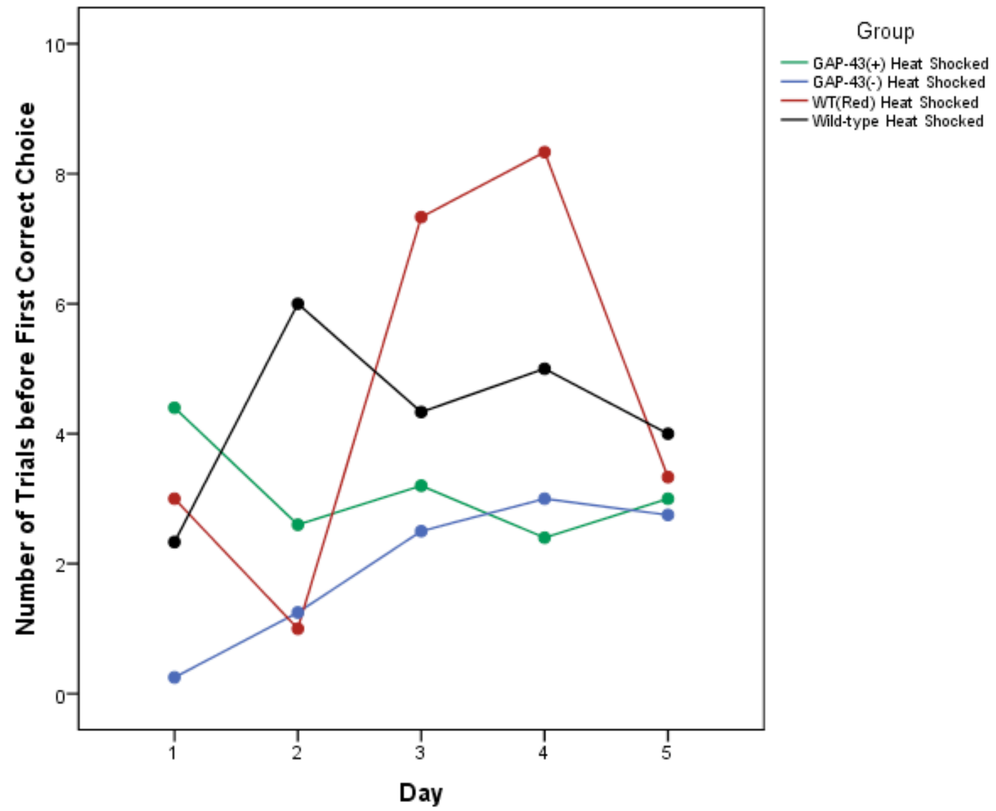

Figure 13. Comparison of heat-shocked fish on the number of trials that passed each training day before the first correct choice was made.

(M = 6.00) before making a correct choice than GAP-43^{-} fish (M = 1.25), *t*(6.69) = 3.03, *p* = .020 and WT^{red} fish (M = 1.00), *t*(6.95) = 2.86, *p* = .025. There were no other significance differences between groups of heat-shocked fish on any of the training days (all *p* > .05).

The Average Latency to Enter the Correct Chamber on Training Days

A mixed design ANOVA revealed no significant interaction between day, group, and heat-shock treatment for the average latency to enter the correct side, *F*(12, 112) = 0.931, *p* = .519, as is illustrated in Figure 14. Similarly, there were no significant differences in latency among heat-shock groups on any of the training days, *F*(12, 56) = 1.36, *p* = .212, as is illustrated in Figure 15. Neither analysis revealed any significant main effects or two-way interactions (all *p* > .05).

The Percent Shift of Time Spent in the Correct Chamber from the Initial to Final Preference Test

Between-groups design ANOVAs were used to analyze data collected from the initial and final preference tests. There was no significant two-way interaction and no significant main effect for group among sub-groups in the increase in percent of time spent in the correct chamber from the initial to final preference tests, which was calculated as the percent of time spent in the correct chamber during the final test minus the percent of time spent in the correct chamber during the initial test (all *p* > .05). These data are illustrated in Figure 16. In the same vein, there was no interaction and no main effect for group

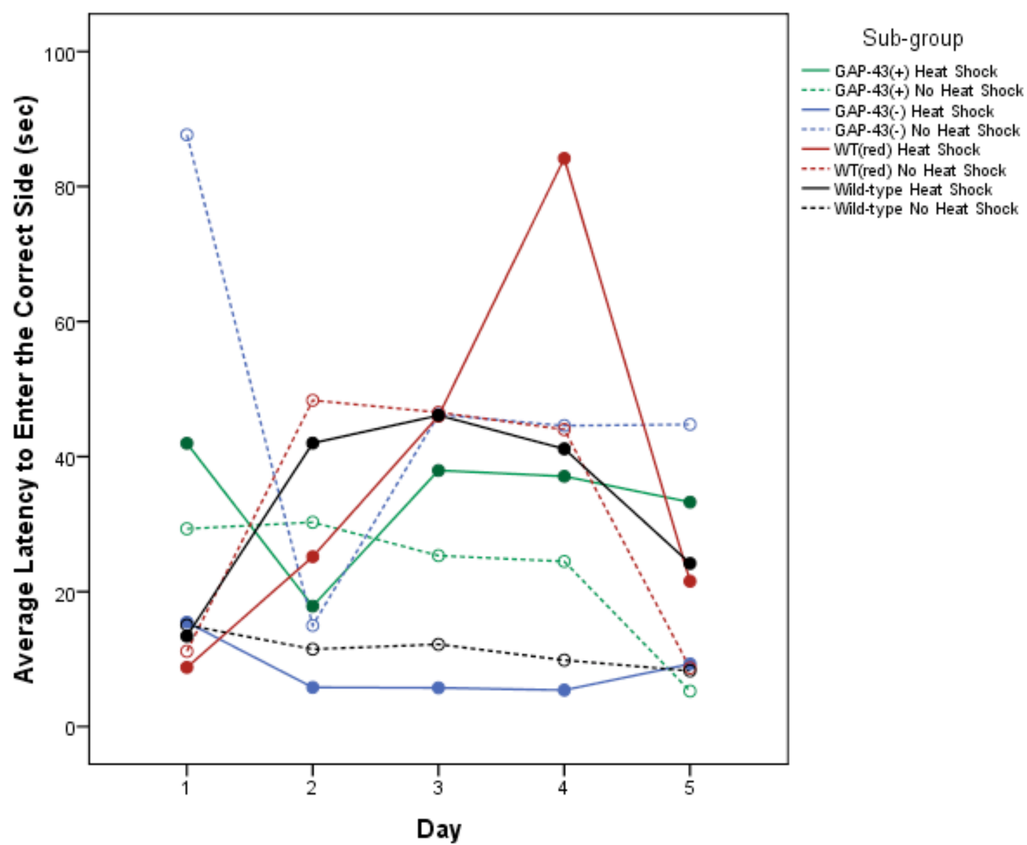

Figure 14. Comparison of eight sub-groups on the average latency to enter the correct side over five training days.

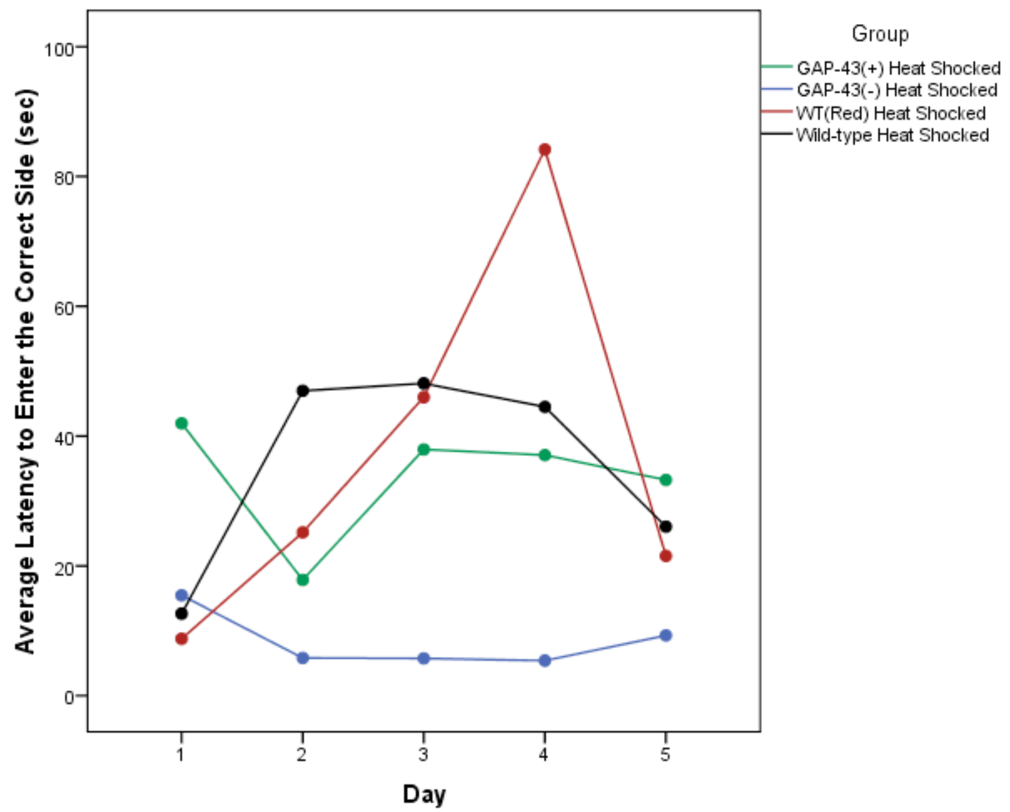

Figure 15. Comparison of four heat-shock groups on the average latency to enter the correct side over five training days.

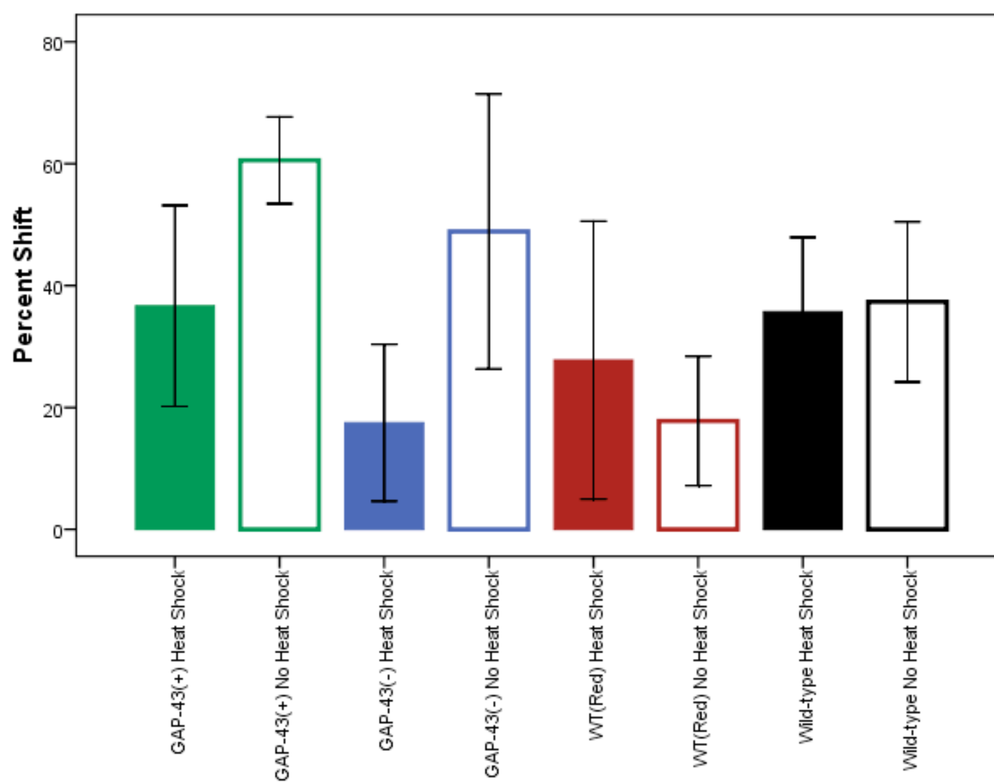

Figure 16. The percent shift of time spent in the correct chamber from the initial to the final preference test (final test – initial test).

among sub-groups in the first 60 seconds of the preference tests, illustrated in Figure 17, or in the 60 seconds immediately following each fish's first choice in the preference tests, illustrated in Figure 18 (all $p > .05$).

However, these analyses did reveal a difference between heat-shocked and not-heat-shocked fish, regardless of group. Not-heat-shocked fish increased the percent of time spent in the correct side during the first 60 seconds of the preference tests (M = 40.28) significantly more than heat-shocked fish (M = 4.03), $F(1, 28) = 5.21$, $p = .030$. This difference between heat-shocked and not-heat-shocked fish is illustrated in Figure 19. A *t*-test revealed that there was no significant difference between heat-shock treatment groups in the percent of time spent in the correct chamber in the initial preference test, $t(34) = -0.75$, $p = .460$. However, the performance of the heat-shock treatment groups is significantly different in the final preference test, $t(34) = 2.32$, $p = .026$. Similarly, in the 60 seconds immediately following each fish's first choice, not-heat-shocked fish increased the percent of time spent in the correct side (M = 49.31) significantly more than heat-shocked fish (M = 5.42), $F(1, 28) = 8.19$, $p = .008$. This difference is illustrated in Figure 20. The treatment groups are not significantly different in the initial preference test, $t(34) = -0.64$, $p = .526$, but are significantly different in the final preference test, $t(34) = 3.16$, $p = .003$.

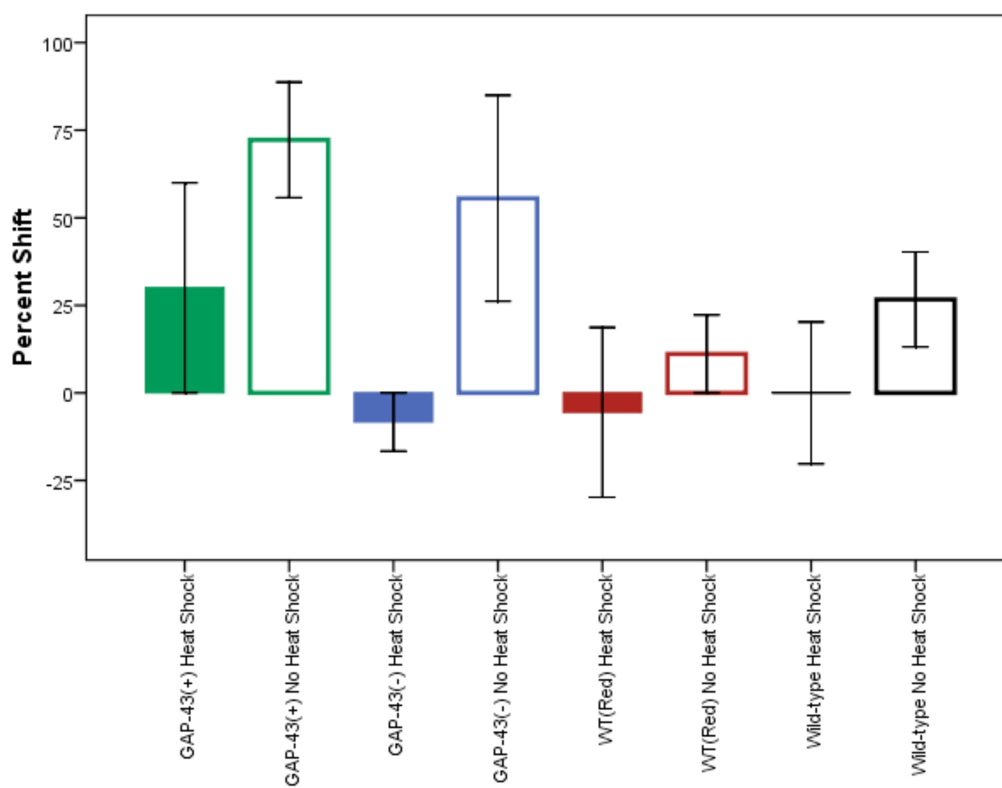

Figure 17. The percent shift of time spent in the correct chamber in the first 60 seconds of the initial and final preference tests (final test – initial test).

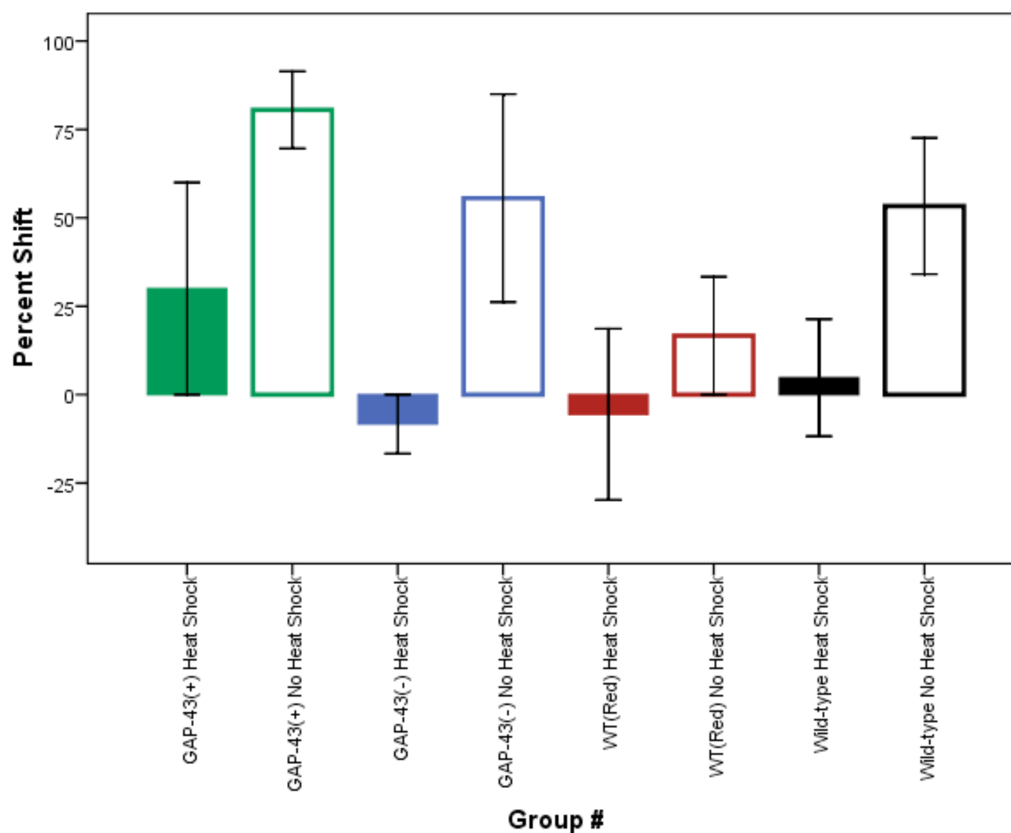

Figure 18. The percent shift of time spent in the correct chamber in the 60 seconds immediately following each fish's first choice in the initial and final preference tests (final test – initial test).

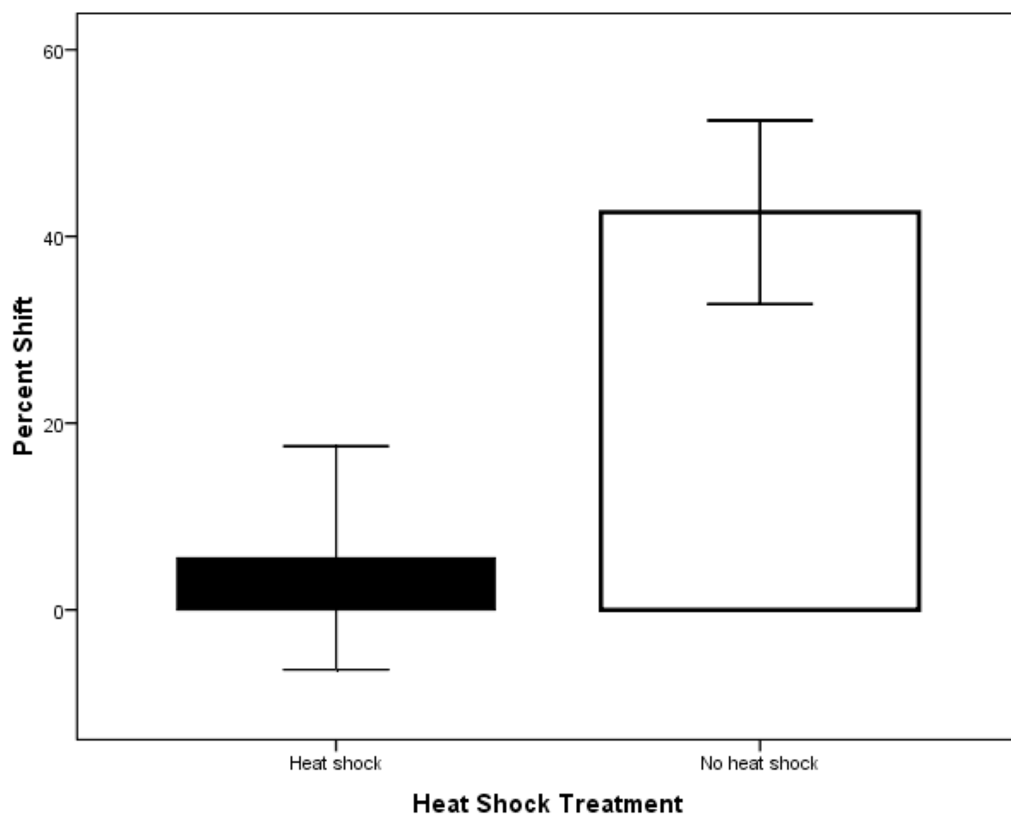

Figure 19. Comparison of heat-shocked and not-heat-shocked fish in the percent shift of time spent in the correct chamber during the first 60 seconds of the initial and final preference tests (final test – initial test).

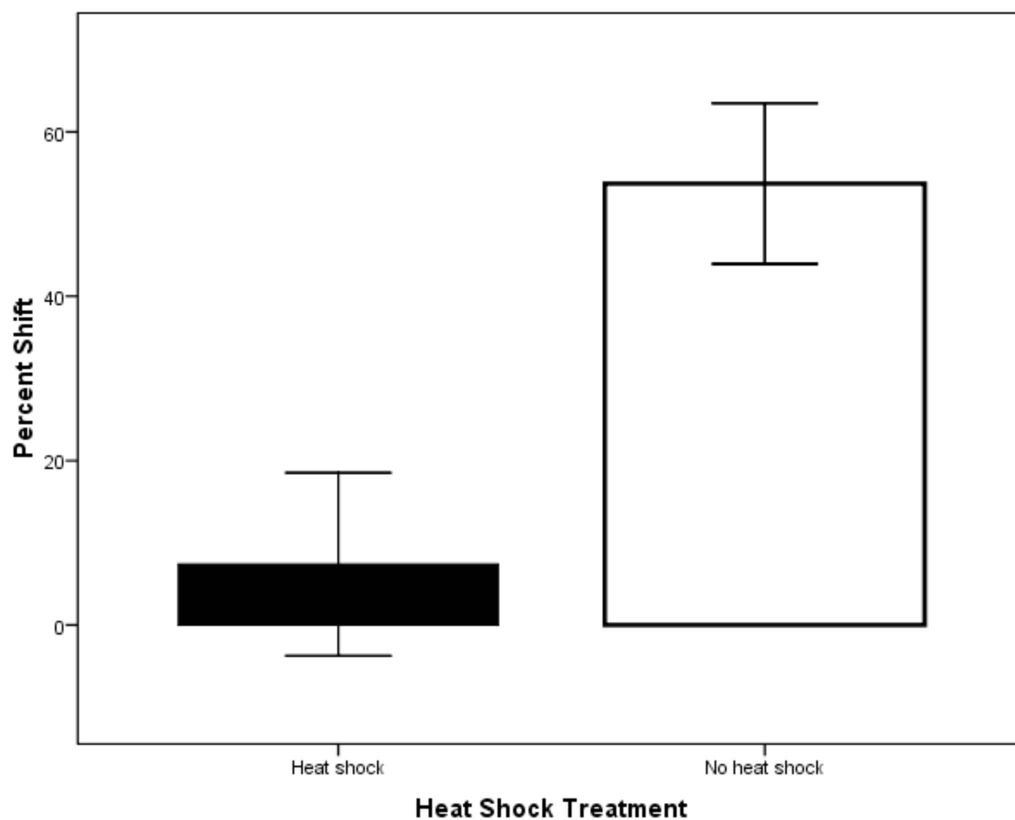

Figure 20. Comparison of heat-shocked and not-heat-shocked fish in the percent shift of time spent in the correct chamber during the 60 seconds immediately following each fish's first choice in the initial and final preference tests (final test – initial test).

When not-heat-shocked fish were excluded from the analysis, a between-groups design ANOVA revealed no significant differences among heat-shock groups in the increase in time spent in the correct chamber from the initial to the final preference test. This result was obtained either when analyzing data from the entire preference test, $F(3,14) = 0.335$, $p = .800$, as is illustrated in Figure 21, or when analyzing the first 60 seconds of each test, $F(3, 14) = 0.509$, $p = .683$, and the 60 seconds immediately following each fish's first choice in each test, $F(3, 14) = 0.561$, $p = .649$. The latter two analyses are illustrated in Figures 22 and 23, respectively. All three analyses revealed no significant main effects (all $p > .05$).

The Number of Fish Entering the Correct Side before Entering the Incorrect Side

A chi-square test showed that there were no significant differences among the eight sub-groups in the number of fish that entered the correct side before entering the incorrect side during the final preference test, $\chi^2(6, N=36) = 3.25$, $p = .777$. There were also no significant differences among the four heat-shock groups in the frequency of fish that entered the correct side first, $\chi^2(6, N=18) = 5.71$, $p = .456$.

The Change in Latency to Enter the Correct Side from the Initial to Final Preference Test

A between-groups design ANOVA revealed no significant interaction between group and heat-shock treatment in the difference in latency to enter the correct chamber from the initial to final preference test, which was calculated as

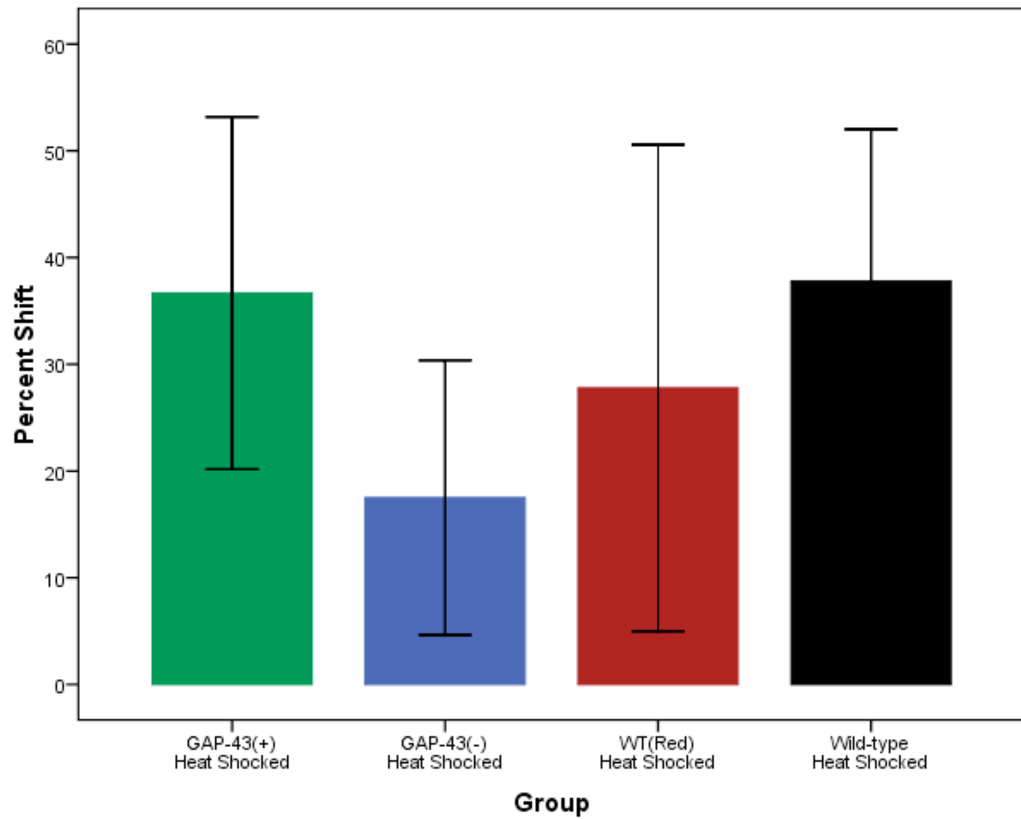

Figure 21. Comparison of heat-shocked fish on the percent shift of time spent in the correct chamber from the initial to the final preference test (final test – initial test).

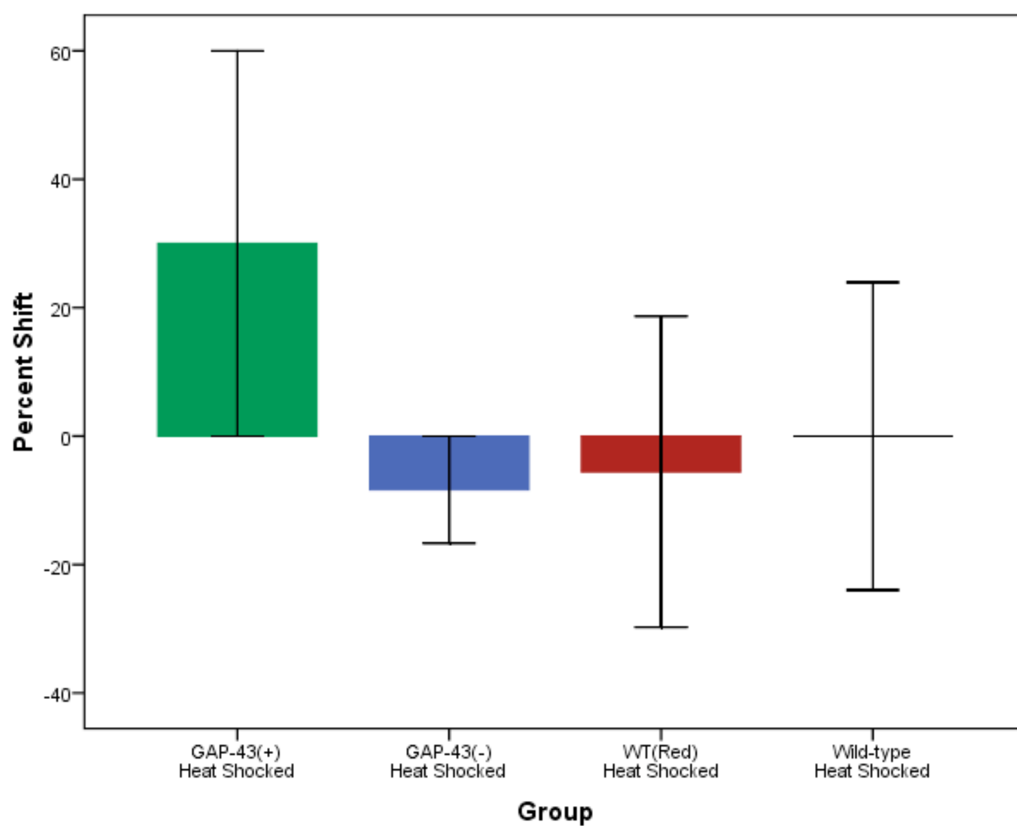

Figure 22. Comparison of heat-shocked fish on the percent shift of time spent in the correct chamber in the first 60 seconds of the initial and final preference tests (final test – initial test).

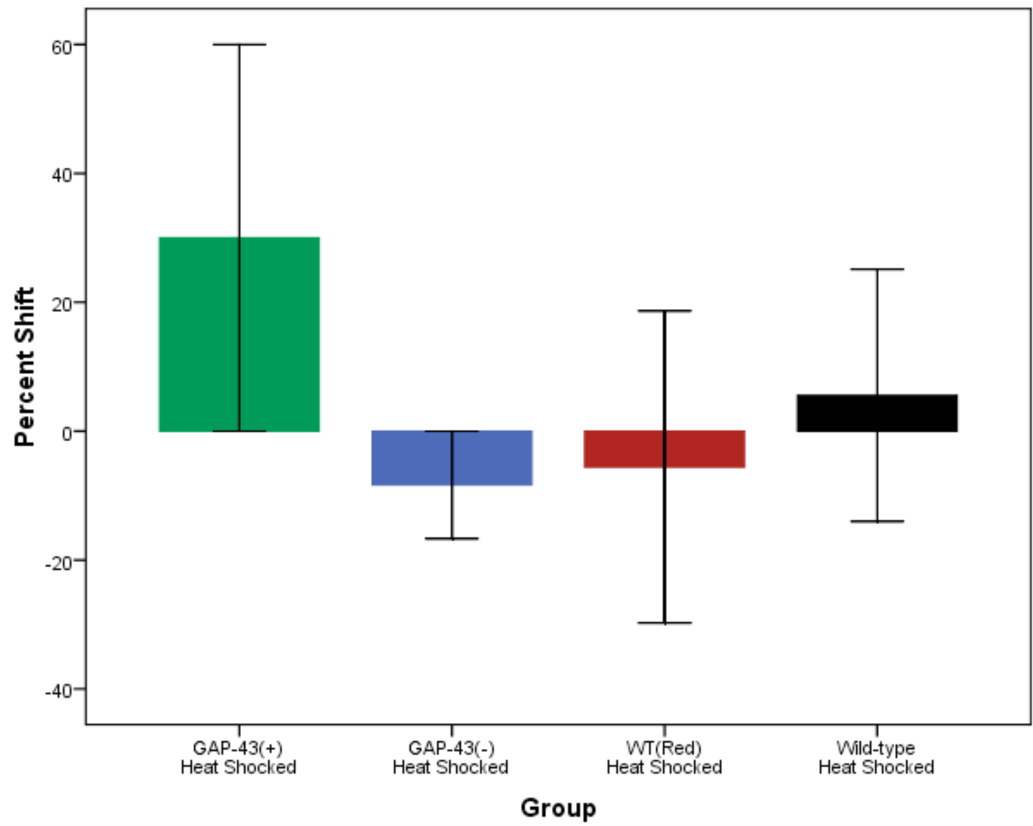

Figure 23. Comparison of heat-shocked fish on the percent shift of time spent in the correct chamber in the 60 seconds immediately following each fish's first choice in the initial and final preference tests (final test – initial test).

the latency to enter the correct side in the initial test minus the latency to enter the correct side in the final test, $F(3, 28) = 1.31$, $p = .292$. Group was not a significant main effect, $F(1, 28) = 5.53$, $p = .026$. These data are illustrated in Figure 24. However, that analysis did reveal that, compared to not-heat-shocked fish, heat-shocked fish showed significantly greater improvement in latency (M = 72.83) to enter the correct side; that is, heat-shocked fish tended to have shorter latencies to enter the correct chamber in the final preference test compared to their latency to enter the correct chamber in the initial preference test than did not-heat-shocked fish (M = 189.89), regardless of group, $F(1, 28) = 5.53$, $p = .026$. The difference between heat-shocked and not-heat-shocked fish is illustrated in Figure 25.

A between-subjects design ANOVA also revealed no significant differences among the four groups of heat-shocked fish in the difference in latency to enter the correct chamber from the initial to the final preference test, $F(3, 14) = 1.05$, $p = .403$. There were no significant main effects (all $p > .05$). These data are illustrated in Figure 26.

Number of Excluded Fish

Of the 52 total fish tested, 16 (30.8%) were excluded according to the exclusion criteria described above. Of the 25 heat-shocked fish that were tested in all groups combined, seven (28%) were excluded. Of the 27 not-heat-shocked fish that were tested in all groups combined, nine (33.3%) were excluded. Table 2 lists the number of excluded fish for each group and sub-group. A chi-square

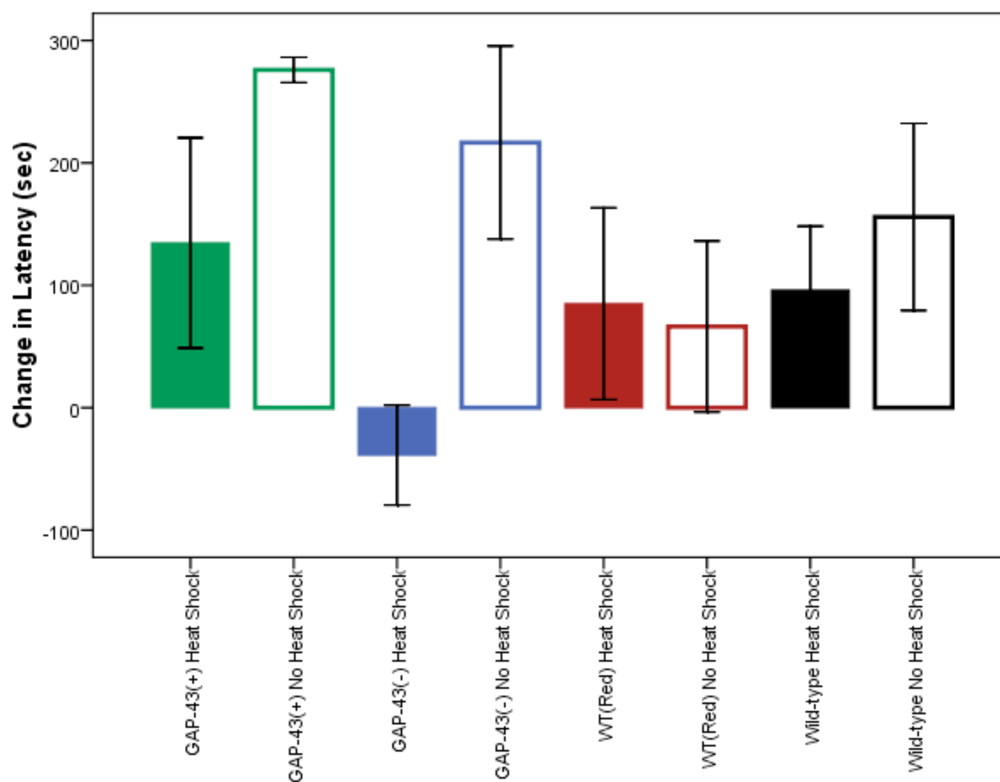

Figure 24. Comparison among eight sub-groups in improvement from the initial to final test in latency to enter the correct chamber (intital test – final test).

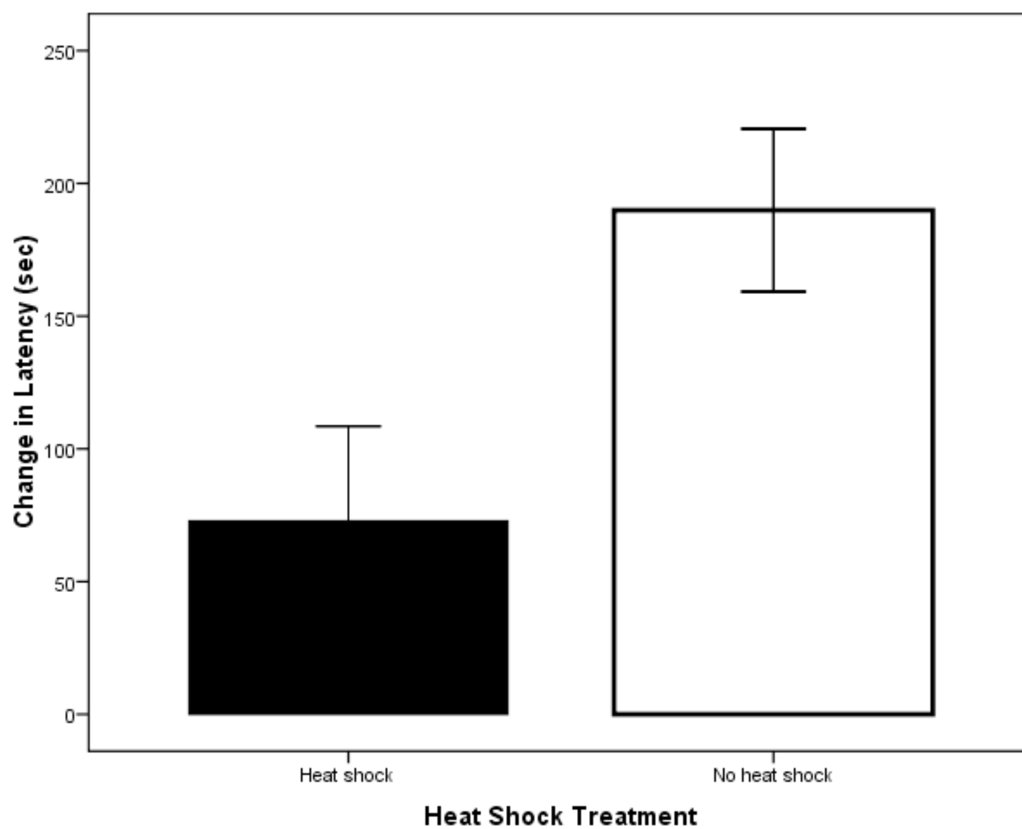

Figure 25. Comparison between heat-shocked fish and not-heat-shocked fish in improvement from initial to final test in latency to enter the correct chamber (initial test – final test).

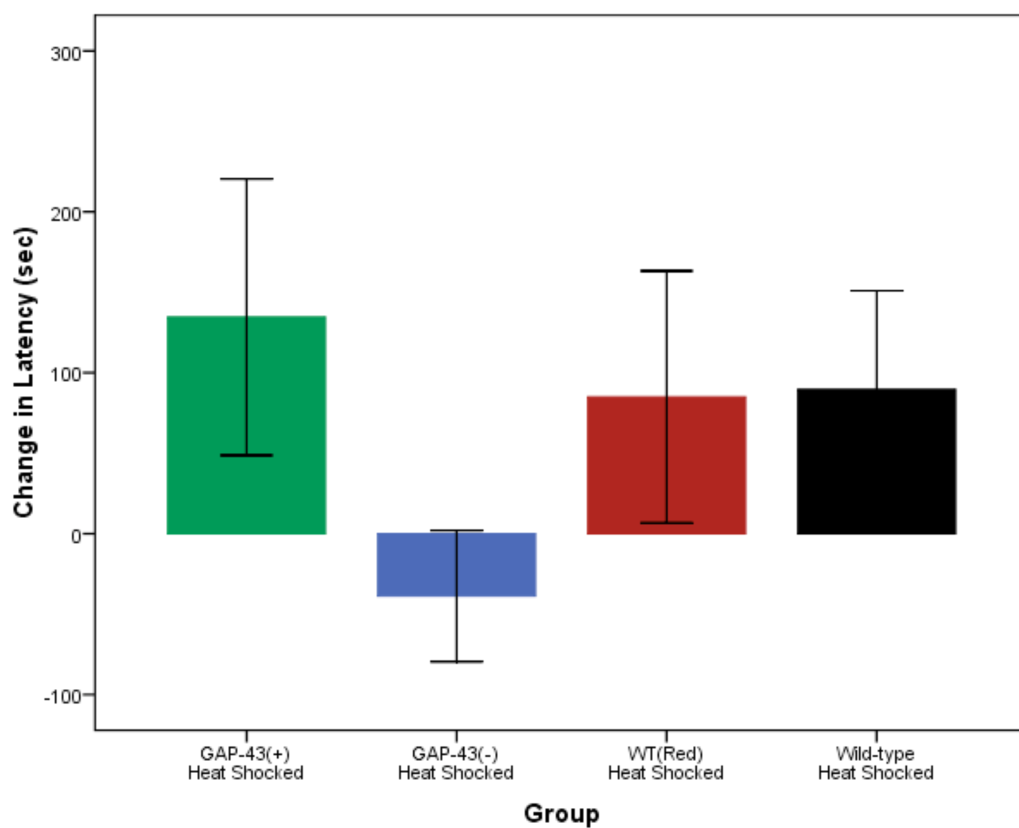

Figure 26. Comparison among four heat-shock groups in improvement from the initial to final test in latency to enter the correct chamber (intital test – final test).

Table 2

Number of Fish Excluded from Groups and Sub-groups

		# fish tested	# fish excluded	% fish excluded	% group excluded
GAP-43^{+}	Heat shock	6	1	16.7%	15.4%
	No heat shock	7	1	14.3%	
GAP-43^{-}	Heat shock	4	0	0	36.4%
	No heat shock	7	4	57.1%	
WT^{red}	Heat shock	8	5	62.5%	57.1%
	No heat shock	6	3	50.0%	
WT	Heat shock	7	1	14.3%	14.3%
	No heat shock	7	1	14.3%	

test revealed that the number of excluded fish from each of the four groups were significantly different from one another, $\chi^2(3, N=52) = 7.96$, $p = .047$. However, the number of heat-shocked fish that were excluded was not significantly different from the number of not-heat-shocked fish that were excluded, $\chi^2(1, N=52) = 0.173$, $p = .677$. In addition to the 16 fish excluded for failing to meet the exclusion criteria, four fish were excluded due to experimenter error and two fish were excluded because they did not eat the food reward.

DISCUSSION

I expected fish that received heat-shock treatment to induce overexpression of phosphorylatable GAP-43 (GAP-43^{+}) to show superior learning abilities on a conditioned place preference task relative to not-heat-shocked GAP-43^{+} fish. This finding would support conclusions from rodent studies demonstrating that the overexpression of phosphorylatable GAP-43 caused a significant increase in spatial learning abilities (Routtenberg, Cantallops, Serrano, & Namgung, 2000). In addition, this finding would suggest that the observed differences in learning were the result of differences in long-term potentiation, rather than the result of developmental differences.

However, the data do not support my hypothesis. During training, heat-shocked GAP-43^{+} fish did not make a significantly higher percentage of correct choices than not-heat-shocked GAP-43^{+} fish, or a significantly higher percentage of correct choices than any other group of heat-shocked fish, on any of the five days. Moreover, when Days 1 and 2 of the assay are combined together and analyzed separately from the rest of the training period, there is still no significant difference in the percentage of correct choices between heat-shocked and not-heat-shocked GAP-43^{+} fish, or between heat-shocked GAP-43^{+} fish and the other groups of heat-shocked fish. Similarly, when performance of fish on Day 2 is isolated and analyzed separately, still no significant difference is found between treatment groups or heat-shocked groups. The treatment groups and heat-shock

groups also did not differ on the average latency to enter the correct side on each training day.

Although treatment groups did not differ on the number of trials that passed before they made their first correct choice on each training day, heat-shocked GAP-43$^+$ fish waited for significantly more trials before making their first correct choice on Day 1 relative to the three other groups of heat-shocked fish.

However, because the screening criterion did not require fish to make any correct choices until the last trial of Day 2, differences in performance on Day 1 and, in some cases Day 2, are often more a measure of what animal behavior researchers call "boldness" (Wilson, Coleman, Clark, & Biederman, 1993) than a measure of learning ability. That is, bold fish tend to explore their environment more readily than shy fish, and thus, in this experiment, would have discovered the food reward earlier than shy fish, allowing bold fish to begin learning the task earlier. Thus, differences in performance that are observed on the first two days of training but on no subsequent days can be considered unimportant as they are a reflection of boldness rather than learning. This is especially true when sample sizes are very small, as they are in the current study, because it is unlikely that equal numbers of bold and shy fish had been assigned to each group.

Heat-shocked GAP-43$^+$ fish also performed identically to not-heat-shocked GAP-43$^+$ fish and all other groups of heat-shocked fish in the final preference test. Heat-shocked GAP-43$^+$ fish exhibited the same amount of

improvement in the percent of time spent in the correct chamber from the initial to the final preference test over a five-minute test period relative to not-heat-shocked GAP-43$^+$ fish and relative to other heat-shock groups. There is also no significant difference found in the percent of time spent in the correct side when analyzing only the first 60 seconds of the preference test, or when analyzing the 60 seconds immediately following each fish's first choice. Furthermore, on average, heat-shocked GAP-43$^+$ fish entered the correct chamber after the same amount of time in the final preference test, and they were equally likely to make a correct choice, as not-heat-shocked GAP-43$^+$ fish and heat-shocked fish from other groups. Thus, heat-shocked GAP-43$^+$ fish did not demonstrate superior learning abilities relative to their not-heat-shocked counterparts or to the other groups of heat-shocked fish.

My second hypothesis was that heat-shocked fish overexpressing an unphosphorylatable form of GAP-43 (GAP-43$^-$) would show either the same or decreased learning abilities when compared to not-heat-shocked fish from the same group. Decreased learning abilities would support previous findings that GAP-43 only increases learning when it is phosphorylatable (Routtenberg, 1985), and also would confirm that the overexpression of GAP-43 in and of itself has no unanticipated effects on phenotype. Conversely, similar learning abilities to not-heat-shocked fish would suggest that when unphosphorylatable GAP-43 is expressed in high quantities, it can interfere with the functioning of endogenous GAP-43 in the cell.

The results show that, in most measures of learning analyzed here, heat-shocked GAP-43$^-$ fish performed similarly to not-heat-shocked GAP-43$^-$ fish. Where the treatment groups did differ, heat-shocked GAP-43$^-$ fish performed better than not-heat-shocked fish, appearing to refute both hypotheses. That is, on the first day of training, heat-shocked GAP-43$^-$ fish made their first correct choice after fewer trials than not-heat-shocked GAP-43$^-$ fish. Also, on the first day of training, the difference between GAP-43$^-$ treatment groups on the percent of correct choices approached significance, with heat-shocked fish making a higher percentage of correct choices than not-heat-shocked fish. However, there were only three fish from the GAP-43$^-$ group that were not heat shocked. On the first day of training, one of those fish made only one correct choice, and the other two fish made no correct choices at all. Thus, the fish in that sub-group had very little or no opportunity to learn the task until Day 2. On the other hand, all four of the heat-shocked GAP-43$^-$ fish made at least one correct choice on the first day, rendering a comparison between the two treatment groups on Day 1 unimportant since such a comparison is, in this case, more a measure of boldness than a measure of learning ability. The misleading result was corrected by analyzing Days 1 and 2 combined, which diluted the effect of fish not choosing on Day 1, and by analyzing Day 2 alone, which eliminated this effect altogether. Both of these secondary measures found no significant difference between heat-shocked and not-heat-shocked GAP-43$^-$ fish.

The GAP-43^{-} treatment groups performed the same on all other measures, supporting the prediction that the overexpression of unphosphorylatable GAP-43 would have no effect on learning. On Days 2, 3, 4, and 5 of training, both heat-shocked and not-heat-shocked GAP-43^{-} fish had the same percentage of choices that were correct and the same number of trials before the first correct choice. Additionally, there is no significant difference between treatment groups on any training day in the average latency to enter the correct chamber.

Analysis of the four groups of heat-shocked fish did reveal a significant difference among groups in the number of trials that passed before the first correct choice was made on certain training days. Heat-shocked GAP-43^{-} fish waited for fewer trials than GAP-43^{+} fish on Day 1. Similarly, heat-shocked GAP-43^{-} fish waited for significantly fewer trials before making their first correct choice than heat-shocked WT fish on training Day 2. However, as was previously discussed, differences observed on Days 1 and 2 of training but not on subsequent days can be considered measures of boldness, not learning, and thus are unimportant.

On all other measures of performance during training days, there is no significant difference between heat-shocked GAP-43^{-} fish and heat-shocked fish from other groups. They displayed similar performances on the percent of correct choices over all five days of training, on the percent of correct choices on Days 1 and 2 combined, and on the percent of correct choices on Day 2 alone. There are also no differences among groups on the average latency to enter the correct side on any of the five training days.

Moreover, there are no significant differences between heat-shocked and not-heat-shocked GAP-43^{-} fish or among heat-shock groups in the final preference test. Treatment groups and heat-shock groups showed the same level of improvement from the initial to the final preference test in the percent of time that was spent in the correct chamber. This result is obtained, either when all five minutes of the preference tests are analyzed, when only the first 60 seconds of each test are analyzed, or when only the 60 seconds following each fish's first choice are analyzed. Furthermore, the latency to enter the correct side during the final preference test was the same in both treatment groups, and both were equally likely to have their first choice in the final preference test be correct. Therefore, when taking all measures into account, there is no difference found between heat-shocked and not-heat-shocked GAP-43^{-} fish, or between heat-shocked GAP-43^{-} fish and heat-shocked fish from other groups.

My third hypothesis was that all WT^{red} fish, namely fish that had a heat-shock inducible, membrane-bound, red-fluorescent protein inserted into their genome, would perform equally well on the conditioned place preference task, regardless of heat-shock treatment. Such a finding would confirm that introducing a membrane-bound protein into a fish's neurons does not have any effect on behavioral phenotype. The results support this hypothesis; that is, there are no meaningful differences between heat-shocked and not-heat-shocked WT^{red} fish on any of the measures. That is, comparisons between heat-shocked and not-heat-shocked WT^{red} fish, as well as between heat-shocked WT^{red} fish and the

other heat-shock groups, reveal no significant differences in the percent of choices that were correct on all five training days, the percent of choices that were correct on Days 1 and 2 combined, or the percent of choices that were correct on Day 2 alone. There are also no differences between treatment groups or between heat-shock groups in the average latency to the correct chamber on all five training days. In addition, improvement from the initial to the final preference test was not different between treatment groups in the percent of time spent in the correct chamber during the five-minute preference tests, during the first 60 seconds of each preference test, or during the 60 seconds immediately following each fish's first choice in the preference tests. Finally, there are no significant differences in improvement in latency to enter the correct chamber from the initial to the final preference test or in the number of fish whose first choice was correct in the final preference test.

Furthermore, there is no significant difference between heat-shocked and not-heat-shocked WT^{red} fish in the number of trials that passed before the first correct choice was made. However, there are significant differences among heat-shock groups. On Day 2, heat-shocked WT^{red} fish waited for fewer trials than WT fish before making their first correct choice. Because this trend is not observed in any other variables, it is likely due to differences in boldness or a Type I error. Therefore, taken as a whole, the results show that heat-shocked WT^{red} fish performed equally well on the learning assay as not-heat-shocked WT^{red} fish, and also performed equally well as other groups of heat-shocked fish.

Although WT^{red} fish performed equally well on the assay as the other groups of heat-shocked fish, there are differences within the group of heat-shocked WT^{red} fish across training days. Heat-shocked WT^{red} fish waited for significantly more trials before making a correct choice on Day 4 than they did on Days 2 and 5. The reason for this considerable deterioration of performance on Day 4 is unclear. The three fish in this group were all trained in different replications, so Day 4 for each fish was a different date. Furthermore, they were all trained by different experimenters on Day 4. Thus, the trend was not caused by a single event or experimenter error. Because the group only contained three fish, the poor performance of all three fish on Day 4 was probably coincidental.

My fourth and final hypothesis was that all wild-type fish (WT) would perform equally well on the assay, regardless of heat shock treatment. Because the WT fish carried no transgenes, heat-shocked and not-heat-shocked WT fish were not different genetically. Thus, any differences observed between WT treatment groups can be attributed to the experience of the heat-shock procedure (37°C hot water bath) versus the heat-shock control procedure (25°C cool water bath).

This hypothesis is partially supported by the data. On most measures, the treatment groups and heat-shock groups are not different; however, there are two variables on which the heat-shocked WT fish performed significantly worse than the not-heat-shocked WT fish. On Days 2 and 3 of training, heat-shocked WT fish waited for more trials before making their first correct choice than not-heat-

shocked WT fish. Additionally, on Day 5 of training a smaller percentage of heat-shocked WT fish's choices were correct. The same trend approaches significance on Day 2; however, when Day 2 is analyzed separately the effect is lost. These differences could be explained by the fact that the temperature changes required for the heat-shock procedure were inherently more stressful to the fish than the lack of temperature changes in the heat shock control procedure. However, it is interesting that this trend is not seen in analyses of the preference tests or in WT^{red} fish, which were expected to show the same behavioral phenotypes as WT fish. This discrepancy could be explained by differences in sample size: the WT group had six fish in each treatment group, whereas the WT^{red} group had only three fish in each group. High inter- and intra-fish variability may be preventing the effect of the stressful heat-shock procedure from being seen in samples of only three fish.

There is also one measure in which WT fish performed significantly worse than heat-shocked GAP-43^{-} fish and heat-shocked WT^{red} fish. On training Day 2, WT fish waited for significantly more trials than GAP-43^{-} fish or WT^{red} fish before making a correct choice. However, because this trend is only seen on Day 2, it can be attributed to differences in boldness.

On all other measures, there are no significant differences between heat-shocked and not-heat-shocked WT fish or among heat-shock groups. During training, both treatment groups made the same percentage of correct choices on Days 1, 3, and 4. Similarly, heat-shocked WT fish made the same percentage of

correct choices as other groups of heat-shocked fish on all five training days. When Days 1 and 2 are combined together, and when Day 2 is analyzed alone, there also are no differences in percentage of correct choices between treatment groups or among heat-shock groups. On training Days 1, 3, and 5, heat-shocked WT fish made their first correct choice after the same number of trials as not-heat-shocked WT fish, and the same was true for heat-shocked WT fish on Days 1, 3, 4, and 5 relative to other heat-shock groups. Both treatment groups and all four heat-shock groups also had the same average latency to enter the correct chamber on each training day.

Heat-shocked WT fish improved the same amount as not-heat-shocked WT and other groups of heat-shocked fish in the percent of time they spent in the correct side, either during the full five-minute preference tests, during the first 60 seconds of each test, or during the 60 seconds immediately following each fish's first choice in the tests. All groups also had similar numbers of fish that entered the correct chamber as their first choice in the final preference test, and showed the same improvement from the initial to final preference in latency to enter the correct side. Taken as a whole, the majority of measures show no difference between treatment groups, and the few differences that are observed were probably caused by small sample size, not by differences in learning ability.

As previously discussed, the heat shock procedure was inherently more stressful to fish than the heat-shock control procedure because it involved not only large changes in water temperature but also relatively rapid changes. Moreover,

even when the temperature was steady, being in 37° water was stressful for the fish. Of course, the hot water bath was designed to be stressful; if the stress levels of the fish had not surpassed a certain threshold, the heat-shock-mediated mutations, which were controlled by a stress-response promoter, would not have been induced. However, it seems that the heat shock procedure was so stressful that it considerably impaired the performance of heat-shocked fish. In the final preference test, not-heat-shocked fish from all four groups combined spent an average of 49% more time in the correct chamber in the 60 seconds immediately following their first choice than they had in the initial preference test. Conversely, heat-shocked fish from all groups combined spent an average of 5% more time in the correct chamber in the 60 seconds following their first choice than they had in the initial preference test. A similar difference is found when examining the first 60 seconds of the preference tests. Furthermore, in the final preference test, not-heat-shocked fish entered the correct chamber an average of 178 seconds faster than they had in the initial test, while heat-shocked fish entered the correct chamber an average of just 68 seconds faster than they had in the initial test. In all three cases, the difference between heat-shocked and not-heat-shocked fish is statistically significant.

The deterioration of performance of heat-shocked fish seems to be directly related to the number of heat-shock treatments received. While there are distinct differences between the performances of the treatment groups in the percent of time spent in the correct chamber in the final preference test, which occurred after

7 days of heat-shock or no-heat-shock treatment, there are no significant differences between these treatments on any measure in the initial preference test, which occurred after only one heat-shock or no-heat-shock treatment.

Considering the results as a whole, there are no meaningful differences among the eight sub-groups. In fact, the only meaningful difference is that heat-shocked fish do significantly worse than not-heat-shocked fish in the final preference test, regardless of group. From these data, three possible conclusions could be drawn. First, one could conclude that the overexpression of phosphorylatable GAP-43 truly has no effect on long-term potentiation. It would follow, then, that the improved learning abilities seen in rodents overexpressing GAP-43 were the result of developmental differences, not the result of improved long-term potentiation. However, considering the small sample sizes and the significant variation in performance caused by the heat-shock treatment in the current study, this first conclusion would be premature.

A second possible conclusion is that the GAP-43$^+$ mutation did have an effect on learning as expected and the GAP-43$^+$ fish did display an increased learning ability, but the assay was not sensitive enough to detect this subtle difference because of small sample size and high levels of variation. A third possible conclusion is that the GAP-43$^+$ fish did have improved ability for long-term potentiation, but the effect this ability would have on learning was completely overcome by the stress caused by the heat-shock procedure and, thus, there were no learning improvements actually displayed.

To determine which of these conclusions is correct, the sample size of each sub-group would need to be increased considerably. It would also be important to reduce variation, both within and between sub-groups. One of the major sources of variation in this assay stemmed from the requirements for inclusion in the study. As already discussed, the criteria for a fish's inclusion in the study were that it make at least two choices, either correct or incorrect, on Day 1, and that it make at least one correct choice by the end of training on Day 2. Because fish were not required to choose correctly on the first day, some fish discovered the food reward and, presumably, began learning the association as early as the first trial on Day 1, while other fish did not discover the food reward until, at the latest, the last trial on Day 2. As a result, some fish performed poorly in the first two days of training, but that performance was not a reflection of their learning abilities. This generated substantial variation in Days 1 and 2 of training, which may have concealed any displayed differences in learning, and which led to numerous misleading findings.

To prevent this problem in the future, the exclusion criteria could be changed to require each fish to make at least one correct choice within the first five trials of the training session on Day 1. This change would ensure that all fish have the opportunity to learn the task beginning half-way through Day 1 at the latest. Presumably, fish who were better learners would then perform better in the remaining five trials of Day 1 and on Day 2 than fish with inferior learning abilities, and thus meaningful data could be gathered from all five days of

training. The disadvantage, however, of this stricter exclusion criteria would be that many more fish would be excluded in each replication. For this reason, changing the exclusion criteria would be practical only if there were a large number of fish available for testing. As a compromise, the criteria could be changed to require fish to make at least one correct choice by the end of the training session on Day 1. This change would result in fewer fish being excluded and, although it would not reduce variation on Day 1, it would help to reduce variation on Day 2.

Another source of variation in the assay was the use of chumming on Days 1 and 2, followed by the absence of chumming on Days 3, 4, and 5. Chumming, namely the process of adding water that smells strongly of shrimp to each of the side chambers, was employed on Days 1 and 2 to encourage fish to leave the safety of the center chamber and thus discover the food reward. However, if the tanks were chummed on all five training days, fish could become dependent on chumming as a cue to start searching for food. Such dependence would become a problem in the final preference test when the tanks are not chummed—when the partitions were raised for the final preference test without the chambers being chummed first, fish may fail to go to the correct chamber because they did not receive the cue to begin searching for food. In contrast, when chumming is stopped on Day 3, it allows fish to learn to use the raising of the partitions as a cue to search for food, which facilitates their performance in the final preference test when chumming is absent.

The disadvantage of chumming only on Days 1 and 2 is that, on Day 3, some fish are dependent on chumming as a cue to search for food, but that cue is absent. Thus, in some cases they do not make any choices for several trials on Day 3 because they have not received the cue to begin searching for food. This poor performance can continue until the fish become hungry enough to search for food despite the absence of the chumming cue. Consequently, the absence of the chumming cue causes a noticeable decrease in performance on Day 3 for some fish but not others, which increases variation within groups on Day 3 and thus makes it more difficult to find significant differences between groups.

A third source of variation in this assay was the stress of the heat shock procedure, to which heat-shocked fish were exposed seven times. The assay could be made more sensitive if the difference in stress between heat-shocked and not-heat-shocked fish were reduced. To achieve this, the heat shock procedure could be modified to be less stressful for fish. Perhaps the simplest modification would be to increase the temperature of the experimental tanks. In the current study, the water temperature in the experimental tanks, which were the tanks that the fish lived in during the experiment, was kept at 25°C. This temperature could be increased to 30°C without any adverse effects on the fish. This simple change would lessen by 5° the change in temperature that heat-shocked fish experience every day, thereby lessening stress.

A second modification to reduce variation would be to shorten the length of time fish are kept in the hot water bath and decrease the temperature of the hot

water bath. The length and temperature of the heat-shock procedure used in this experiment were chosen because they resulted in optimal expression of the inserted trangenes (A. Udvadia, personal communication, February 3, 2009). However, sacrificing optimal expression of the trangenes in favor of a shorter, cooler, and less-stressful heat-shock procedure may produce more meaningful results.

Another way to reduce the stress associated with the heat-shock procedure would be to refrain from heat shocking fish on the days of initial and final preference tests. Because the effect of the stress is directly related to the number of times the fish are heat shocked, eliminating two days of exposure to the heat shock procedure could reduce variability between the treatment groups. Originally, it was decided to heat shock fish on all seven days to facilitate good performance on the final preference test by making the events on the test day match as closely as possible those of the training days. In addition, it was thought that the mutations in the transgenic fish might reach peak expression between 24 and 48 hours after each heat shock exposure (A. Udvadia, personal communication, February 3, 2009), and thus the mutations would be in full effect only on Day 1 of training if the fish were heat shocked the day before, which is the day of the initial preference test. However, this second reason has been found to be invalid as it is now known that peak expression of the mutation occurs within 24 hours of the heat shock exposure (A. Udvadia, personal communication, April 1, 2009). Alternatively, to reduce the number of times the fish are exposed

to the heat shock procedure, the assay could be shortened by eliminating one or more of the five training days. However, the disadvantage of shortening the assay is that there may not be sufficient data to determine the learning curve of individual fish.

Finally, in addition to modifying the heat shock procedure to be less stressful, the heat shock control procedure could be made to be slightly more stressful. The cool water bath to which not-heat-shocked fish were exposed was 25°C. The temperature of this bath could be slightly raised to be more stressful to the fish. Preliminary experimentation would be required to determine a temperature that is more stressful than 25°, but that does not induce expression of the trangenes. If the procedure were changed in this way, it would be especially important to analyze the brains of the not-heat-shocked fish post-mortem to ensure the lack of transgene expression.

Although the data collected in the current study were unable to provide any evidence for the role of GAP-43 in long-term potentiation, this experiment will be important in guiding the design of more effective assays to answer this research question in the future. The use of heat-shock inducible mutations was a novel approach to investigating the genetic underpinnings of learning in zebrafish, and the knowledge that the heat-shock procedure significantly changes the learning behavior under study is important. Although the heat-shock procedure was, in the end, too stressful for the assay to detect any differences in learning among groups, there are several ways in which the heat shock procedure could be

modified to be less stressful for fish in future experiments. In fact, much of the variation that may be concealing differences in learning ability in the current study could be reduced or eliminated in future studies through a few simple changes to the structure of the assay. In further pursuit of uncovering the role of GAP-43 in learning, it would be worthwhile to repeat this study with a larger sample size, stricter exclusion criteria, warmer experimental tanks, fewer and less-stressful heat shock exposures, and a more stressful heat shock control procedure.

Although the technique of using heat-shock-induced transgenes in zebrafish to study the role of GAP-43 in long-term potentiation has yet to be perfected, implementing the changes that I have proposed above would be worth the time and effort involved. Zebrafish provide a unique opportunity to separate the role of GAP-43 in development from its role in long-term potentiation and learning. Without this separation, it is impossible to know what aspects of behavior in an adult organism are merely the products of development, and which are being dynamically changed and modified as a result of the organism's interactions with its environment.

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