**The Correlation between Dietary Intake and Agouti-Related Protein Expression in Zebrafish, *Danio rerios***


## Abstract

This study was conducted to examine how selectively induced starvation affects the levels of mRNA for the Agouti-Related Protein (AgRP) in Danio rerios (Zebrafish). AgRP is located in the hypothalamus, and regulates body weight. In order to test this research problem, two groups of fish were utilized. One group faced induced starvation and the other was fed under normal conditions for nine day period. Once the nine days were up, the fish were euthanized and the brain tissue was dissected from all of the fish. The mRNA in the Zebrafish brain was extracted from the tissue with the use of an RNAqueous kit from Life Technologies. The mRNA was then converted to cDNA and used in RT-PCR. Results yielded inconclusive data related to AgRP expression levels in both groups of fish, the experimental and the control. It was concluded that AgRP levels in both groups were found to be extremely low as compared to the expression levels of the 18S control.


## Background for the Research

Obesity has become major problem in the United States, overtaking smoking as the leading cause of death. (Medical News Today) According to justthink.org, childhood obesity has tripled over the last 30 years. Two-thirds of the US population is overweight, and 33.8% of US adults are now obese. This number has increased dramatically over the last 20 years. Obesity can increase the risk of obtaining heart disease, type 2 diabetes, certain types of cancer, and other life threatening diseases. There have been several genes linked to obesity and disorders such as diabetes. One such gene found in humans and many other vertebrates including primates and mice is the Agouti Related Protein. It has been found when AgRP is over expressed the protein can be linked to hyperphagia (excessive over eating) which then leads to obesity (Lin, 2001). AgRP is produced in the brain in the hypothalamus, and its job is to regulate body weight. AgRP production takes place in the AgRP neuron located within the hypothalamus. Leptin and Ghrelin are hormones in the body that inhibit or activate the AgRP for AgRP production. The mechanism for this is as follows.

Before meals, the hormone ghrelin is produced in the lining of the stomach, the fundus. This 28 amino acid peptide and hormone stimulates hunger by binding to the ghrelin receptor on the AgRP neuron. This activates the neuron and allows the AgRP to be produced thus causes the individual to feel hungry. Leptin is a hormone that inhibits appetite and prevents AgRP production. When food is consumed, fat tissue begins to secrete leptin. It is then sent to the AgRP neuron, halting the production of AgRP to be sent to the Melanocortin 3 or 4 receptors. This tells the body that it is satisfied and does not need to take in more food. However,

The Mc3R and Mc4R receptors control metabolism and body weight. AgRP only binds to the Mc3R or Mc4R and signals that the body is hungry. If AgRP levels are too high, it causes excessive over-eating called hyperphagia, and over time, this leads to obesity.

Much research has been conducted about the Agouti Related Protein. Obese humans have a higher concentration of AgRP plasma levels, and obese transgenic mice also have over expression of AgRP in their hypothalamus (Pan, 2005). Another study shows that when mice were fed 20% less of a protein enriched diet, decreasing their diet caused their AgRP gene coding expression to increase as a result (Pillot, 2011). The hormone ghrelins main target in the hypothalamus is the AgRP/Npy neurons and after injecting ghrelin into rats, AgRP mRNA levels rose (Kamegai, 2001). In order to examine AgRP levels in a gene expression analysis, real-time PCR is a necessary tool.

The Zebrafish, or (*Danio rerio*) contains similar organs to Homo sapiens that can be utilized for research. In this study, the use of Zebrafish is necessary because like humans, most of AgRP protein synthesis occurs in the neurons in the hypothalamus within the brain, and in order to view the gene expression of the Agouti Related Protein located in the hypothalamus, the brain tissue must be dissected, purified, converted to cDNA, and analyzed through Real-Time PCR.

## Statement of the Research Problem

The purpose of this study was to determine whether AgRP expression would be increased in fish experiencing induced starvation. It was hypothesized that the Zebrafish experiencing starvation would have an elevated AgRP mRNA expression compared to the fish fed normally. Since the starved fish were consuming less food, less leptin would be secreted from fat tissue, and more ghrelin production would occur while preparing for meals. More ghrelin would then bind to the AgRP/Npy neurons activating the production of AgRP. The starved fish would have secreted less leptin, making it challenging to halt AgRP production. As a result, mRNA levels of AgRP will be higher in the brain tissue of the starved fish.

## Research Design and the Variables

In order to test the hypothesis of this experiment, the following experimental design was set up. Two experimental groups were utilized to test the variable. Tank 1 was the experimental variable, contained the fish being starved, and the experimental control, Tank 2 was fed normally. These two groups were necessary in order to test the variable of starvation. First the Aquarium had to be set up.

## Preparing the Fish Tanks for the Zebrafish:

Two 55 gallon aquarium tanks were set up, and filled with water. In order to condition the water and detoxify the heavy metals that are very harmful to the fish, Tap Water Conditioner was used to remove the chlorine. A safe biological environment was provided with Nutrafin Cycle Biological Aquarium Supplement to introduce bacteria into the new habitat.

The useful bacteria helps break down the ammonia and nitrate build up. Fluorescent lights were utilized as light sources and the timers were set to turn on the lights at 6 AM and turn off at 8 PM. The water temperature was kept at a constant temperature of 25.5 degrees Celsius with a water heater. In order to filter water system, an Aqua Clear power filter was used. Water samples were taken to a local pet store to ensure that the pH level was 7. Once the water was safe, the fish were ready to be acclimated to the water.

Seventy-two male Danio rerios were purchased and shipped from Carolina Bio Supply. The fish were fed under normal conditions to help them become better accustomed to their new environment. Many were lost do to stress conditions. Once the fish population became stable, 44 Zebrafish were divided into the 2 tanks with 22 fish in each. The 9 day experiment was then ready to begin.

The average zebrafish mass was approximately .5 grams. Each fish in Tank 1 was fed 2% of their body mass, and each fish in Tank 2 was fed 4% of their body mass. The following calculations were made to determine the amount of food to feed each tank. Food adjustments were made and recalculated every 3 days when 5 fish were removed from each tank.

Tank 1: .5g x 22 fish x .02 = .22g of food for the tank each day. The fish were fed twice daily, .11g per feeding.
Tank 2: .5g x 22 fish x .04 = .44g of food for the tank each day. The fish were fed twice daily, .22g per feeding.

**Fish Euthanization**

At the end of the 3 day period, 5 fish from each Tank 1 were put into a beaker filled with 500mL of water, and 5 fish from Tank 2 were put into a beaker filled with 500 mL of water. The chemical MS-222 (fish anesthetic) from Western Chemical was utilized to euthanize the Zebrafish humanely after the 3 day period. .10g of MS-222 powder was weighed out at and was placed into the water. The final concentration was .2mg/mL, just overdosing the fish enough to put them to sleep. The fish were then dried with lab tissues and wrapped in cling wrap and placed into a gallon zip lock labeled with the corresponding tank. The bags were put into a foam storage box filled with dry ice to preserve the fish. They were stored in the freezer maintained at a constant -20 degrees Celsius.

For Days 4-6 the fish in each were fed one-third less of the quantity they had been fed on a daily basis to adjust the amount for the fish that had been removed. Tank 1 was fed .073 g of food twice daily for a total of .146 g per day, and Tank 2 was fed .147 g of food twice a day for a total of .294 g of food per day. At the end of this feeding cycle, the same Fish Euthanization was followed as written above. For the final fish feeding cycle, the Zebrafish were fed two-thirds of the original amount. Tank 1 was fed .037 g twice daily for a total of .074 g per day, and Tank 2 was fed .073 g twice daily for a total of .146 g per day. The same Fish Euthanization was followed again.

**Dissection Process**

After all the fish needed in the experiment had been euthanized, the brains of the Zebrafish were to be removed and placed into sterile and RNA free eppi tubes. The brain of a Zebrafish runs linear with the length of their body. In order to dissect the brain from the fish, a sterile pipette tip was sent through the body horizontally to provide a way to stabilize the fish, and to separate the upper and lower portions of the body. Then, an incision was made starting between the eyes going length wise down the fish. The brain is located between the eyes of the fish in a gray membrane. A few more scrapes were made to free the brain tissue from the membrane, and then the brain was placed in the proper eppi tube. In order to extract the AgRP mRNA out of the hypothalamus tissue, the RNAqueous-4PCR Kit from Applied Biosystems was used. Much prep work was needed to prepare the lab for the extraction, because the lab area must be completely RNase free.

**Data Collection Procedures**

To ensure that the equipment and surroundings are Ribonuclease (also known as RNase) free, the lab tables and equipment must properly be cleaned. RNases cut mRNA into smaller pieces. In order to halt the RNase enzyme from cutting, RNase OUT is used to bind the enzyme from cutting. Green Cleaner was sprayed onto the lab tables and surrounding area and then washed off with a sponge until no suds remained. This step was repeated twice. Then, bleach at a 30% concentration was sprayed onto the tables and cleaned with a sponge until suds no longer remained. Culligan water was used to cleanse the sponges. Bleach was added a second time, and powder-free latex gloves were required when washing again. After the bleach process was complete, a new set of gloves were put on, RNase out was sprayed on them and spread with a lab tissue. RNase OUT was sprayed onto the table and dispersed over the table with a special new RNase out sponge. The lab area was then RNase free. Then, the equipment to be used in the lab, consisting of; a heating plate, a centrifuge, pipettors, RNase free pipettor tips, and all the solutions necessary for the RNA extraction, were cleaned with RNase OUT on lab tissues, and then placed into the lab area. Gloves were frequently changed to ensure that the equipment was not contaminated with RNase. Each time a glove was changed; more RNase OUT was applied to them. After the brains were dissected, they were ready for the mRNA extraction process. The protocol from Applied Biosystems was used to extract the mRNA.

After the mRNA was extracted from the tissue in the hypothalamus, it needed to be converted to cDNA because mRNA is unstable and to be used as template DNA for the Assay. This process was referred to as reverse transcription.

After the mRNA has been converted into cDNA, it was taken out of the PCR machine and stored in the freezer until it was needed for the final PCR reaction. The concentrations of cDNA in each eppi were taken with the NanoDrop2000.

1 µL of DNA was put onto the NanoDrop. A laser was sent through the DNA to detect the concentration. The concentration then appeared on the computer screen after the concentration was measured. The average concentration of the DNA was around 2.25 µg/µL.

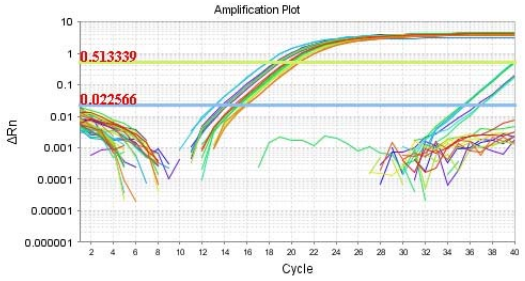
**Setting up the Gene Expression Assay**

The reagents necessary for the gene expression assay were; 10 µL TaqMan Gene Expression Master Mix, 1 µL of the AgRP gene expression assay or the 18S Control gene expression assay, 1 µL of the template DNA, and 8 µL of nuclease-free water. The DNA needed to be diluted to a lower concentration of .02µg/µL. C1V1calculations were made in order to determine the amount of DNA and EB Buffer to place into the DNA dilution. 20 ng is needed per reaction so only 1µL has to be added to each well.

Gloves were worn during the entire process to avoid DNA contamination, and the reactions were kept on ice until they were ready to be run in the RT PCR machine. Once the plates were filled, they were run with a Comparative CT Quantitation Analysis with a fast ramp speed. The plates were run under one cycle of 95 degrees Celsius for 10 minutes, then 40 cycles of 95 degrees Celsius for 15 seconds and 60 degrees Celsius for 1 minute, and lastly an infinite hold of 4 degrees Celsius. The gene expression quantitation was run and the data was collected onto the computer.

**Results**

After the data was collected onto the computer, the results showed that it was inconclusive to determine whether the induced starved or the Zebrafish fed under normal conditions had higher mRNA AgRP levels. The curve that amplified first in all of the plates was the 18S control. They began to amplify around cycle 15. This had a very high gene expression because the gene is always regulated. It is always expressed because it codes for the ribosomes essential for every cell. Second group of curves was the AgRP gene expression. They had a much lower level of expression compared to the 18S, and did not even begin to start amplifying until the 35th cycle. The 18S gene expression has a definite curve and proves that the conversion from brain tissue to mRNA to cDNA was quite successful.




**Discussion and Conclusions**

The data collected in this experiment did not support what was hypothesized in the beginning because it was impossible to determine which group of fish had higher AgRP expression levels from the results. However it was concluded that AgRP gene expression levels in the brain tissue are extremely low compared to the 18S ribosomal mRNA levels. Since ribosomes are necessary in all cells, the gene is up regulated especially since ribosomes are so essential to cellular function. If one were able to specifically dissect the hypothalamus from the brain tissue, it's possible that AgRP levels could be higher. The ability to examine the AgRP levels directly in the hypothalamus could provide more conclusive evidence to determine the expression more accurately that helps regulate body weight. Also, after analyzing the results, it was evident that the concentration of AgRP specific cDNA per PCR reaction was relatively low because it took nearly 30 cycles before any amplification took place in the AgRP gene expression. So, AgRP expression levels could not be effectively measured. Even though AgRP levels could not be determined, it shows that the brain was properly dissected and the mRNA was properly extracted and converted into cDNA. For further research, the cDNA should not be diluted down as much to ensure a higher concentration for the gene expression assay, making it possible to have enough amplification to recover results. Again, this was probably due to the fact that AgRP is only expressed in the hypothalamus and therefore, since the hypothalamus is an extremely small portion of the overall volume of the brain, the relative expression of AgRP compared to other genes would be low.

Another factor is that only the mRNA levels were being examined in the gene expression assay, not the protein levels, and there is a possibility these could differ from one another. One messenger RNA molecule can code for multiple proteins copies to be produced and thus the levels might not directly correlate to one another. Also, due to costs of supplies, it's challenging to have enough supplies and time to do hundreds of reactions to be sure of the validity of the results. Lastly, it's a challenge to determine exactly how much each individual fish consumed because they were all in a tank with one another. Some fish could be more aggressive than the other and get to the food faster than the less aggressive fish. The aggressive fish would then get to intake more food than the other fish. Perhaps in further research, the fish could be more severely starved over a longer period of time, or maybe tissue cell culture in order to examine the AgRP levels more closely.

This research shows promise towards finding the relationship between diet and the expression of the Agouti Related Protein levels in the brain. If this relationship can be established, it could provide useful information in the study of Human Obesity.