EFFECTS OF CARBAMAZEPINE ON THE BEHAVIOR AND COLORATION OF SIAMESE FIGHTING FISH (*BETTA SPLENDENS*)

A Report of a Senior Study

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ABSTRACT

The use of pharmaceutical drugs has increased over the past one hundred years due to many advances in the health field. This, however, comes with an unintended environmental impact. Although they may work well on humans, they can be detrimental to animals that live in areas where these drugs are being taken. Humans will naturally excrete these drugs after their use, and some of these drugs cannot be filtered out by water treatment plants because of their small size. An example is Carbamazepine (CBZ), an anticonvulsant used to treat bipolar disorder and epilepsy. CBZ is one of the most abundantly found chemicals in waterways in every corner of the globe. So many of these animals, large or small, that live in water runoffs that have CBZ present in them are absorbing it into their bodies constantly. Siamese Fighting fish or "Betta fish" (*Betta splendens*) live in the wild in many of these affected areas. Betta fish are also naturally very territorial and aggressive to the same sex in the wild and in captivity. This study's aim was to see how the aggression levels and vibrancy of the fish were affected by exposure to CBZ. This was done by exposing the fish in a paired test design to 1.0µg/L CBZ for 20 days (10 days exposed and 10 days control). This study found that the aggression of the fish was not significantly affected ($p= 0.139$), but the change in the vibrancy due to the CBZ vibrancy was significant reduced ($p=0.045$). Interestingly, the females had higher levels of aggression than the males ($p=0.00036$) which was not an expected outcome. Future studies could use this study as a template with the addition of more fish to sharpen the effects of the CBZ. This study could also be an example for future studies

if the goal was to look at the effects of other pharmaceutical drugs on betta fish or the effects of pharmaceuticals on other animal species that live near waterways that aren't exclusively fish.

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CHAPTER I

INTRODUCTION

Pharmaceutical drugs have been one of the most influential developments for increasing the lifespan of humans. The purposes of the over 20,000 pharmaceutical drugs have a very broad range that cover everything from inflammation to being a stimulant to the central nervous system (CNS) when needed. Depending on the pharmaceutical, each one has a geographic area where it is ingested heavily which can vary from one country to the entire globe. No matter what the drug's sphere of influence is, the path is similar across the board when dealing with pharmaceuticals. After absorption and action, all drugs are eliminated as waste. After excretion or being thrown out, pharmaceuticals enter waterways after passing through municipal waste treatment facilities. Indeed, pharmaceutical drugs including caffeine, nicotine, acetaminophen, and many others have been detected in each continent (Wilkinson 2022). While sewage treatment stations can get most large molecules out, some of the small molecules that make up these drugs can get past it. Therefore, whatever animals live in these run-off waterways experience effects of substances that were not designed for them.

Background of Some Pharmaceuticals

Many chemicals occur naturally and can infiltrate the waterways in that manner, but in the case of pharmaceutical drugs, these synthetically made chemicals do not occur naturally. Some examples of these drugs, as shown in Table 1, for human consumption include stimulants, pain relievers, anticonvulsants, biguanides, calcium-channel blockers, antibiotics, and anti-inflammatory drugs to name a few.

Caffeine is an example of a stimulant of the CNS since it blocks the nerve receptors for adenosine which slows nerve activity, and simultaneously increases nerve activity all together (Sitzman 2013). Traces of caffeine are found in many places in the Unites States like in multiple waterways in Pennsylvania (Loper 2007). Caffeine has shown to be a troublesome contaminant when introduced to animals like the *Corbicula fluminea* (Asian clam), as caffeine causes natural biomarkers and stress levels to increase (Aguirre-Martinez 2015). Nicotine, a stimulant like caffeine, is also prevalent in global waterways coming in seventh in overall abundance (Wilkinson 2022).

There are also pain relief medications like acetaminophen (commonly known as Tylenol) that are used to regulate the body's temperature when dealing with high fevers, and the degradation of the fever leads to less pain. Acetaminophen has been found in slightly less concentrations than caffeine in Pennsylvania *(*Loper 2007). Animals that normally live in very specific temperatures could potentially be adversely affected by this since they need that heat to survive.

Table 1: Examples of some frequently occurring pharmaceuticals that are found in most continents and their uses.

Name/Type	Found/Abundance	Effects	Source(s)
Stimulant			
Caffeine	USA/Global	Psychoactive Central Nervous System (CNS) stimulant	Wilkinson 2022; Princeton UNIV; Aguirre-Martinez 2015; Loper 2006; Sitzman 2013
Nicotine	USA/Global	Simultaneous Adrenaline and Dopamine release	PNAS (Wilkinson 2022); NIH;
Pain Relief			
Acetaminophen (Tylenol)	USA/Global	Regulates temperature (usually fever) via control of the hypothalamus; Blocks prostaglandin synthesis (the initiator of inflammation)	Wilkinson 2022; Mayo Clinic; Loper 2006
Anticonvulsant			
Carbamazepine	USA/Global 0.02-1.0 µg/L	Controls Epilepsy and Bipolar Disorder	Wilkinson 2022; Mayo Clinic; Aguirre-Martinez 2015; Loper 2006
Biguanides			
Metformin	USA/Global	Assists with the control of Insulin (usually in diabetic patients)	Wilkinson 2022; Mayo Clinic;
Ca-Channel Blocker			
Verapamil	USA/Global	Treats chest pain and angina caused by cardiac arrythmia (irregular heartbeat); Treats hypertension (high blood pressure)	Wilkinson 2022; Chen 2018; Kania 2015
Antibiotics			
Ciprofloxacin	USA/Global	Broad Spectrum Antibiotic used treat UTIs (Urinary Tract Infections), pneumonia, skin and bone infections, and other similar bacterial infections	Wilkinson 2022; Johnson 2015; Kenyon 2022
Metronidazole	USA/Global	Antibiotic used to treat skin infections, rosacea, and oral infections including infected gums and dental abscesses	Wilkinson 2022; Cox 2009;
Anti-Inflammatory			
Ibuprofen	USA/Global	A NSAID (Non-Steroidal Anti-Inflammatory Drug) used to treat mild to moderate pain	Aguirre-Martinez 2015; Chopra 2020;
Antihistamine			
Diphenhydramine	USA/Global	An inhibitor of histamine release which is used to lessen the duration of asthmatic symptoms	Wilkinson 2022

Carbamazepine, an anticonvulsant used for the treatment of epilepsy and bipolar disorder is found in similar concentrations to caffeine in the Pennsylvania study (Loper 2007), and it also was one of the main drugs that helped cause the natural biomarkers and stress levels to increase in the Asian clam study (Aguirre-Martinez 2015).

Metformin is also a pharmaceutical drug that helps control the body's insulin flow in diabetic patients that was found in many global waterways (Wilkinson 2022). In most cases, Metformin is prescribed to diabetic patients when exercise alone has not been enough to keep their health on the right track. Normally, improper use of this drug can happen in very large amounts due to human body size, but for smaller organisms, this improper use range of metformin is much smaller. One large side effect of improper use of metformin is lactic acidosis which is where the body makes an excess amount of lactic acid. Extremely high levels of lactic acidosis can potentially lead to death in large and small organisms.

Verapamil is a pharmaceutical drug that targets the calcium channels to help patients with chest pain and angina caused by cardiac arrythmia (irregular heartbeat). It is also able to treat hypertension in some cases. It was found in multiple waterways across the globe (Wilkinson 2022), and in a test to see the effects of verapamil on Siamese fighting fish in Poland, Verapamil was found to cause the fish to have an abnormal decreased amount of aggression to external stimulants (Kania 2015). Verapamil has even been shown to be effective in fighting Mycobacterium tuberculosis because of its calcium-channel disrupting capabilities (Chen 2018), and since calcium-channels are a key piece in muscle contraction, it is possible that it could have harmful side effects on other organisms.

Of the many types of antibiotics available, ciprofloxacin and metronidazole are two that are found in abundance globally (Wilkinson 2022). Ciprofloxacin was found to be very

close to the toxic range for surrounding wildlife in European rivers, and the same study found that the mixture of multiple types of antibiotics like ciprofloxacin can take it over the toxic threshold (Johnson 2015). Another global survey found that ciprofloxacin was higher than the toxic threshold in multiple sites around the world other than Europe, and this high concentration has led to an increase in antibiotic resistant E. coli (Escherichia coli) (Kenyon 2022). Metronidazole, the other antibiotic mentioned, was found to be in live brine shrimp *(Artemia*) in large concentrations (Cox 2009). Brine shrimp live in multiple global saline locations, high concentrations of the pharmaceutical would be caused by the influence of metronidazole in those waters.

Similarly, ibuprofen, a widely known anti-inflammatory drug, has been noted as an environmental contaminant that is growing in its environmental abundance (Chopra 2020). Ibuprofen is used to slow the body's natural production of prostaglandins which cause inflammation and pain due to a stimulus. This inflammatory process is a natural part of the healing process the body takes. If other animals are contaminated with ibuprofen in their water and other sources, they could potentially be lowering their inflammatory response when either injured or under attack by a disease or infection. In a large organism, this inflammation allows for white blood cells to flood the area (Schottenfeld 2006). This lack of natural inflammatory response in a small organism's body could lead to death as their body's natural immune system would be impaired.

Diphenhydramine, commonly known as Benadryl, is an over-the-counter antihistamine that is found all over the world has also been investigated recently as a potential environmental toxin (Berninger 2011). Diphenhydramine acts as a histamine blocker in the body. Histamines help cause inflammation, they dilate blood vessels, they

increase heart rate after contractions of the muscles, and they assist with gastric acid secretion (Aymard 2012). In a human, larger volumes of this drug that are ingested may not have a large affect to their health, but to a smaller organism, the ingestion of this drug on any amount could potentially cause a detrimental effect on their homeostasis.

Pharmacokinetics and Pharmacodynamics

Each drug that is ingested by the body moves through the body and is metabolized. Two terms used to describe pharmaceuticals in the body are pharmacokinetics and pharmacodynamics. Pharmacokinetics is the study of how the drug is absorbed by the body, how the body spreads the drug and metabolizes the drug, and how the body excretes the excess drug. Pharmacodynamics, on the other hand, is the study of the mechanism of action of the drug and how that concentration affects the therapeutic ability of the drug (Marino 2023). It also studies the body's overall short term and long-term reactions to the drug. Each drug, depending on its intent, has different methods for their pharmacodynamics and pharmacokinetics, but they all are absorbed, metabolized, and excreted in the end.

As an example, the drug carbamazepine is used to treat epilepsy and bipolar disorders along with other brain disorders (Loper 2007). The first step for the pharmacodynamics of carbamazepine revolves around the inhibitory neurotransmitter GABA (gamma-aminobutyric acid) which regulates the levels of dopamine and glutamate in the brain. Disorders like epilepsy and bipolar disorder normally have low levels of GABA (which can cause apoptosis), but carbamazepine acts as a GABA agonist meaning that it behaves like GABA. This would mean that patients receiving carbamazepine would then be able to regulate the levels of dopamine and glutamates in their brains. This is because carbamazepine acts as a stabilizer for the voltage gated sodium channels on neurons in the brains, and those channels

play a key role in the regulation of neurotransmitters (Ayano 2016). Carbamazepine, however, can lead to side effects like nausea, skin rashes, bowl issues, decreased bone marrow functions, and many other side effects depending on the patient according to the National Library of Medicine.

Regarding the pharmacokinetics of carbamazepine, it is taken orally via chewable or non-chewable tablets that are taken with each meal or 2-4 times a day in general. The drug is metabolized in the liver using hepatic enzymes and the quick metabolism of the carbamazepine. Carbamazepine then enters the bloodstream from the liver, the drug travels throughout the body by staying suspended in the blood plasma. After its normal cycle, whatever is not absorbed and possibly the decaying carbamazepine, the drug is passed through the kidneys which filters it out of the blood as it is then excreted through urine. Only a very small amount (1%) is excreted through fecal matter. Carbamazepine has a bioavailability of 80% in the body. This is achieved when the maximum blood plasma levels for carbamazepine is reached after 2-8 hours in the body. In a single dose of carbamazepine, the half-life of the drug in the plasma can reach around 18-54 hours, but after multiple doses, the half-life can decrease to 10-25 hours. This decrease in the half life is due to the increase to the increased hepatic metabolism and the increased metabolism of the carbamazepine itself (Ayano 2016).

Uses of Carbamazepine

Each of the drugs in Table 1 has been found in waterways around the world because of the human body's ability to metabolize and excrete said drugs. Carbamazepine is just one example of a drug that has infiltrated global waterways in high amounts. According to the Proceeding of the National Academy of Sciences (PNAS) in 2022, carbamazepine was the

most detected pharmaceutical drug in rivers and bodies of water around the world (Wilkinson 2022). This high volume of carbamazepine found around the world means that there is a large amount of people that are using the drug. Carbamazepine is available to humans via prescription in most countries, and can be taken orally in either 100 mg, 200 mg, 400 mg, or 100 mg/mL supplements. Carbamazepine is used as an anticonvulsant for the control of epilepsy and bipolar disorders. It can stop or slow polysynaptic responses in the brain and slow the potentiation of the neurotransmitters. This potentiation in the human brain is when the strength of the synapse signal becomes stronger overtime. When there is an abnormal amount of potentiation a general neurotransmitter activity in the brain, it can result in the symptoms of epilepsy, bipolar disorders, and a few other illnesses.

Carbamazepine also has a high excretion rate compared to other pharmaceutical drugs. According to the FDA (United States Food and Drug Administration) and a test done by Novartis, of the Carbamazepine that was ingested, 72% of the original ingested amount was fond in the urine and 28% was found in the feces (Novartis 2009). Of the carbamazepine that was ingested, all of it was excreted after a metabolization time. Similarly, in the same test, only 3% of the original carbamazepine that was ingested was unchanged, and the rest had been metabolized into another metabolite of carbamazepine. Regarding the high volume of carbamazepine that is excreted after use, according to a recent study, less than 10% of the carbamazepine found in the waterways are absorbed at water treatment plants (Hai 2018). This magnitude of excretion when taking carbamazepine coupled with the very small amount of it that is taken out of the water in waste treatment plants could be the reason that is the number one pharmaceutical found in waterways around the world.

Abundance of Carbamazepine Found in Waterways

Carbamazepine has been found at varying concentrations across global waterways. A few examples of these contamination areas and their abundances are the 0.4 ppb (0.4 µg/L) found in Minnesota (Carbamazepine in Drinking Water 2011), around 0.02 µg/L consistently found in Pennsylvania (Loper 2007), and the 0.08-1.0 µg/L found all over the world (Wilkinson 2022). There was also a normal abundance of 0.1 µg/L found in the groundwater of Canada, Japan, USA, and Germany along with one sample region in the UK that ranged from 0.425-3.6 µg/L (Hai 2018).

Background of *Betta splendens*

Siamese fighting fish (*Betta splendens)*, also called betta fish, are useful models for fish in scientific research. They have been used in behavioral, endocrinological, genetic, and developmental studies in the past. Siamese fighting fish usually show high levels of aggression towards one another when in separated tanks in visible range. Studies have shown that Siamese fighting fish tend to have a higher level of aggression, in males and females, when they are constantly in an isolated tank rather than with multiple other subjects (Iwata 2021). Even though both sexes showed aggression, males were notably more aggressive than females. Females would also view this level of aggression in the males when looking for a mate.

Vibrancy is an indicator of the health of a fish, including betta fish. In colonies and schools of fish social stressors like mating competition can increase the pigmentation of the skin (Border 2018). General stressors like the diet and environmental conditions of fishes can also change the pigmentation of the skin (Kiswara 2022). When dealing with fish that are being kept as pets, the housing conditions of those fish can even be slightly affected by the housing environment that they are in (Dolan 2015).

Even though their conspecific behavior has been studied immensely, betta fish are still used in many experiments like studies that test endocrine disruptors in fish (Dzieweczynski 2012). They have also been used in other studies to test how chemicals like fluoxetine can affect their behavior (Dzieweczynski 2012) and one study where 17α-ethinylestradiol was tested to see how it would affect the reproductive systems of male and female Siamese fighting fish (Forsatkar 2021).

Since Siamese fighting fish have been used in many different experiments in the past, they can be useful in seeing the effects that carbamazepine can have on fish in the wild. The aim of this study is to see whether the anticonvulsant drug carbamazepine can alter the behavior and vibrancy of Siamese fighting fish, and therefore, possibly other aquatic species.

CHAPTER II

MATERIALS AND METHODS

A total of 10 betta fish (*Betta splendens*) (5 males and 5 females) were used for the experiment and housed in 3.5 L tanks each purchased from PetSmart (Alcoa, TN) however, the tanks were only filled with 1 gallon of water each. The water that was used was created by adding about 3 mL of pond started to 4-5 gallons of tap water. Each fish was added to their tanks and labeled with a divider placed in between each tank so that the fish could not see each other and potentially become aggressive. The fish were fed once daily with betta fish pellets also purchased form PetSmart (Alcoa, TN). The first five fish exposed to CBZ were kept in exposure tanks for the first 10 days which allowed for two experimental days for data gathering (one each five days). The fish were then switched so that the exposed fish became the control, and the control fish became he exposed. They were then kept in their tanks for another 10 days again allowing for two days for data gathering.

Before the experiment began, the 1.0 µg/L concentration had to be prepared to be added to the experimental tanks. Creating the same concentration needed a total of 0.014329g Carbamazepine (CBZ) per 1.0 mL of 95% ethanol. The weight of the CBZ was calculated to ensure a 1.0 µg/L concentration in the gallon of water for the tanks (1 gal = 3.785 L). The CBZ can only be successfully dissolved in ethanol. However, to ensure the fish

were not heavily affected by the added ethanol, only 0.5 mL of the solution would be added to the experimental tanks. This required a total of 0.085974g of CBZ to be added to 3 mL of 95% ethanol (0.014329g CBZ per 0.5 mL 95% ethanol). For each of the control tanks, 0.5mL of 95% ethanol was added without any CBZ to take a bias factor caused by the ethanol out of the experiment.

For each of the testing days, a vibrancy test and a behavior test were performed for each of the fish. The vibrancy test was done by comparing a picture the previous of the initial test to the current state of the fish. The vibrancy was graded on a scale from -5 to 5 where -1 to -5 was a decrease in vibrancy, 1 to 5 was an increase in vibrancy, and 0 was no change in the vibrancy. Other helpers were used to measure the vibrancy of the fish during the vibrancy tests. The behavior tests were performed once before the addition of the CBZ and ethanol. Each behavior test was done by taking one tank at a time and placing it on the lab table for easier viewing. The mirror was added to the end of the tank that the fish was facing, and when the mirror was placed a timer was started. Using the timer, the behaviors of each fish was viewed and written down for every 20 second increment for five minutes. As represented in Table 2, each behavior would either be scored a 0 for non-aggressive behavior or a 1 for aggressive behavior.

Data Analysis

All comparisons between exposed and unexposed fish were analyzed using MS Excel using paired t-tests.

Table 2: Ethogram used during the aggressive behavior tests.

H	Hover- hangs in water column with fins not spread.	0
BR	Bottom rest.	0
B	Breathing (surfacing and gulping air)	0
FS	Fin Swim- slow swimming using pectoral fins; without fins in spread condition	0
SS	Serpentine Swim- rapid, uses entire body and S-shaped movements without fins in spread condition.	0
S	Shaking- shimmies the body, usually with fins and gills spread.	1
GS	Gill Spread- only indicate this if the behavior is not done as part of one of the next behaviors.	1
FSH	Fin Spread with body Horizontal to mirror. This may include periods of gill spreading as part of the action pattern.	1
FSP	Fin Spreading display with body Perpendicular to the mirror. This may include periods of gill spreading as part of the action pattern.	1
A	"Arches" body. This may include periods of gill spreading as part of the action pattern.	1
Ch	Charges mirror- approaches rapidly but does not touch. This may start from FSP. A charging fish may have its fins and gills spread. A movement towards the mirror out of a display makes the behavior a charge.	1
Ct	Contacts mirror- same as above but with actual contact. Watch approach before designating as either Ch or Ct. Probably a rare behavior for most fish.	1
L	Leave- swims away from the mirror.	0

Table 3: Sample table used during the aggressive behavior's tests.

Fish #	1	2	3	4	5	6	7	8	9	10
Esposure ?	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)	(Y/N)
Time (s)										
0-20										
20-40										
40-60										
60-80										
80-100										
100-120										
120-140										
140-160										
160-180										
180-200										
200-220										
220-240										
240-260										
260-280										
280-300										

CHAPTER III

RESULTS

Over the course of the three-week experiment, no fish died due to any health risk or researcher error. One fish (fish #2) was not active and did not display similar behavioral tendencies to the other fish and subsequently was not included in the analyses. During the paired test design, the fish that were being exposed to the carbamazepine (CBZ) appeared to have a trend towards being less aggressive compared to the time that they spent in the control tanks as can be seen in Figure 1; however, this reduction was not significant ($p= 0.139$). For each of the individual fish, their baseline test (prior to any exposure) was compared to their average exposure results and average control results to find the average change in both. The average change in the control behaviors was 0.333 points while the average change in the exposure behaviors was -0.722. The females also showed elevated aggression compared to the males during the entire study ($p=0.00036$).

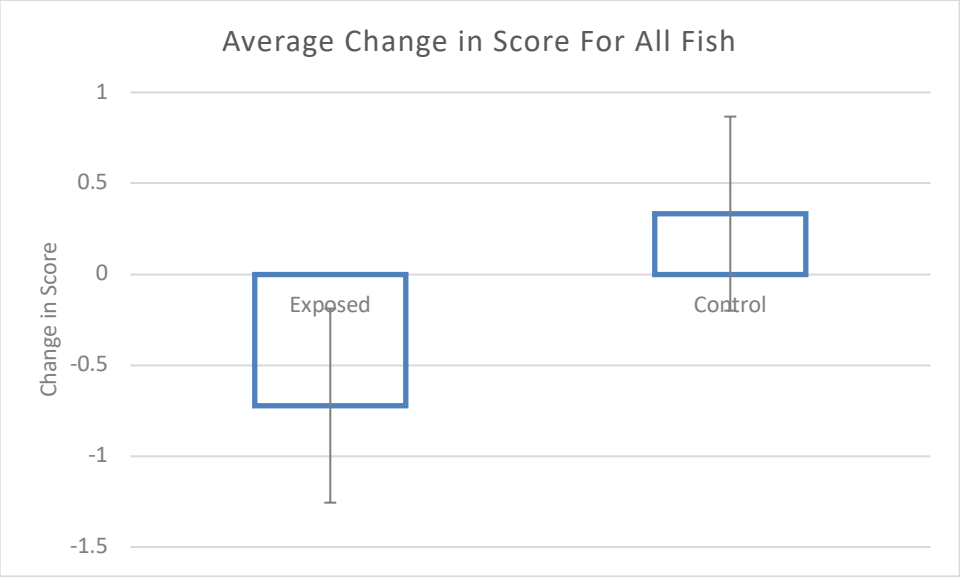


Figure 1: The average change in behavioral score for all fish by taking their individual score changes taken during their time in the exposure period and their control period.

The vibrancy portion of the study encompassed the same principles as the behavioral test as each fish was examined for a change in their vibrancy when during the exposure period and the control period. The vibrancy endpoint did find significant changes even when the behavioral analysis did not. When exposed to the CBZ for the ten-day period, the fish showed a significant reduction in their vibrancy when compared to the change in their changes during their control phase ($p=0.045$). With the baseline being a score of zero for all fish, the average change for all fish was found with the individual averages in score changes from the fish during both phases. As can be seen in Figure 2, the average change in score for all fish during the control phase was only -0.2 points while the average change in score for all fish during the exposed phase -1.1 points. This data for the vibrancy tests was taken using vibrancy scores from all fish including fish #2.

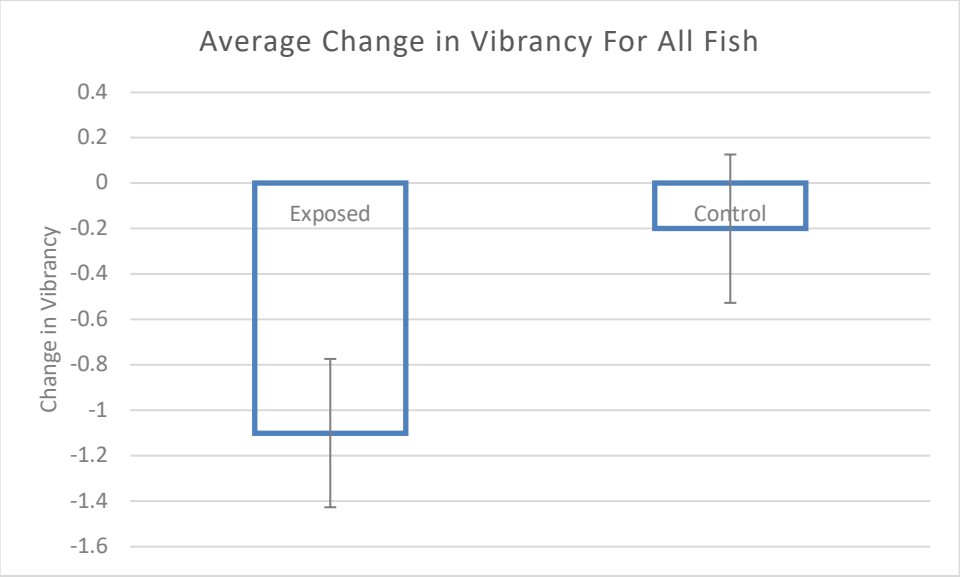


Figure 2: The average change in vibrancy for all fish by taking their individual score changes taken during their time in the exposure period and their control period.

Shown below is an example of how the vibrancy test was scored during the entire experiment. The first image (Figure 3) was taken on the first day (Day 0; September 8, 2023) prior to the exposure of any of the fish. The second image (Figure 4) was taken on the third test day (Day 10; September 18, 2023) after the first phase of the experiment was completed. As can be seen in the photos, Figure 3 shows a much brighter fish #1 compared to a slightly darker, less glossy scaled fish in Figure 4. This change in vibrancy after the first phase of the experiment while fish #1 was in the exposed group resulted in a vibrancy score of -3.

Figure 3: Photograph taken on September 8, 2023, showing fish #1 before exposure to carbamazepine (CBZ). This fish had not been given a score on the vibrancy test yet as there was no previous picture to compare it to.

Figure 4: Photograph taken on September 18, 2023, showing fish #1 ten days after exposure to carbamazepine (CBZ). The vibrancy scored a -3 on the vibrancy test.

CHAPTER IV

DISCUSSION

The effect of carbamazepine (CBZ) on the behavior of betta fish appeared to have no significant impact but there was an effect on the vibrancy of the fish over the course of the study. There are many factors that were in play during the study like the stress the fish were under and the housing that they were in that could have played a role in the differences in the aggression and decrease in vibrancy. Even with the non-significant change in aggression during the test, the carbamazepine did seem to have a trend in decreasing the aggressiveness of the fish which could potentially be seen in similar studies with longer testing periods and increased sample size.

Aggression

Studies have suggested that different sexes of betta fish respond differently to conspecifics. Females and females can be kept together, and males and females can be kept together, but in most cases, if two males are kept in the same tank then they will fight until one of them is left (Pavlus 2020). This would lead to the conclusion that the males would be more aggressive towards the other species. The current study, however, found that the females tended to be more aggressive than the males when in the behavior analysis test. One study found that Japanese medaka (*Oryzias latipes*) experienced increased behavior when

confronted with higher numbers of males or females (Grant 2002). If there were more males with fewer females, then the males would show higher amounts of aggression and similar results happened when the inverse ratios were studied. However, since these betta fish were isolated, it is unlikely that there was any cause for error due to social interactions. Another possible reason for these high levels could be that these specific females just happened to be naturally more aggressive than their male counterparts. At least four of these females could also have been reproductively active which could have spiked their aggression levels (Holder 1991). Another possibility for the differences in aggression between males and females specifically could have been because the CBZ treatment had a more pronounced effect on the females. There is limited data on the differences in aggression between male and female betta fish, but depending on the species there can be varying differences in the sex-dependent aggression. Further studies would need to be performed on the effects of CBZ on sex-specific aggression or just on sex-specific aggression for betta fish.

The behaviors of betta fish in this study have similar trends as in another study where batta fish were exposed to Fluoxetine, a serotonin inhibitor. That study found that after a short exposure to Fluoxetine, the fish experienced a decrease in aggressive behaviors (Lynn 2007). This, however, would be hard to compare to the effects of CBZ as both drugs are different and take on entirely different pathways. The visual aspects of the study could also have played a role in the differences in the behaviors of the exposed and unexposed. One study found that betta fish tend to be more aggressive when exposed to a video playback of another fish of the same sex rather than when a mirror is used (Verbeek 2007). Since a mirror was used in this study, this could have possibly led to some of the outlier scores for each fish, and the two-outlier fish altogether.

Vibrancy

The vibrancy of betta fish can be influenced by the health of the individual fish and influenced by the setting that they are in. The vibrancy of betta fish can be influenced by the health of the individual fish and influenced by the setting that they are in (Dolan 2015). However, the amount that these variables changed with the change in tank size and temperature was found without significance. This would make sense as all ten fish were kept in the same environment and in the same amount of water. The diet of the fish also can generate changes in the color and vibrancy of the fish (Kiswara 2022). Since the fish were added to their respective tanks that day that they were taken from the store and given general betta fish food, this could have led to a change in their diet. These fish could have been fed a different betta fish food while they were being held at the store, so their new diet at the beginning of the experiment could have led to some of the changes in their vibrancy. However, this change in their diet could not be the main reason for the larger decrease in vibrancy when exposed to CBZ as it was not significant.

Each individual fish also has their own style of stress coping. A study on Atlantic salmon found that stress did change the color or pigmentation of the skin depending on how vulnerable the individual was to the stress (Øverli 2008). However, since this was a paired design for each of the betta fish, even if each fish showed their own coping mechanism for the stress that they had, it would not explain the significant difference between the discolorations seen when exposed to CBZ. Another study showed that when cichlids (*Cichlidae*) were introduced to a large school of other cichlids, their pigmentation would increase even after a short amount of time (Border 2018). This could explain why the betta fish lost pigmentation due to their isolation from other individuals. However, due to other

factors like territorial protection, it is understood that some betta fish are normally isolated in the wild (Perry 2022). None of these ideas, however, are able to explain why the CBZ had such a significant effect on the vibrancy of the fish other than the added stress that comes with the exposure.

Future Studies

Possible future studies could be done with the blueprint of this study in mind. However, increasing the number of fish in the study could possibly create a larger data set which would give even more accurate results. Considering how close the aggression data was to being significant, there is the possibility that increasing the number of fish in the study could yield significant data. This is because the trend of the data was headed toward being significant, but with such a small sample size, it is hard to gain clear results. The vibrancy may yield even larger results if also ran again in a similar experiment with a larger number of fish.

Further studies could also investigate the effects of CBZ on specific animals that live in different environments and countries. Since wild beta fish are mostly found, or originally found, in southern Asia, it would be interesting to see how other species are affected (National Geographic 2023). CBZ is found in waterways globally, so seeing how it can specifically affect other species of fish and other species of small aquatic animals would be possible, especially in Appalachia where there are multiple species of aquatic animals.

Different chemicals and pharmaceuticals can also be used in other studies to see how they affect betta fish or other small aquatic species. It is recommended that future studies focus on smaller species of animals because those are the ones that are usually the most affected by chemicals and pharmaceuticals.

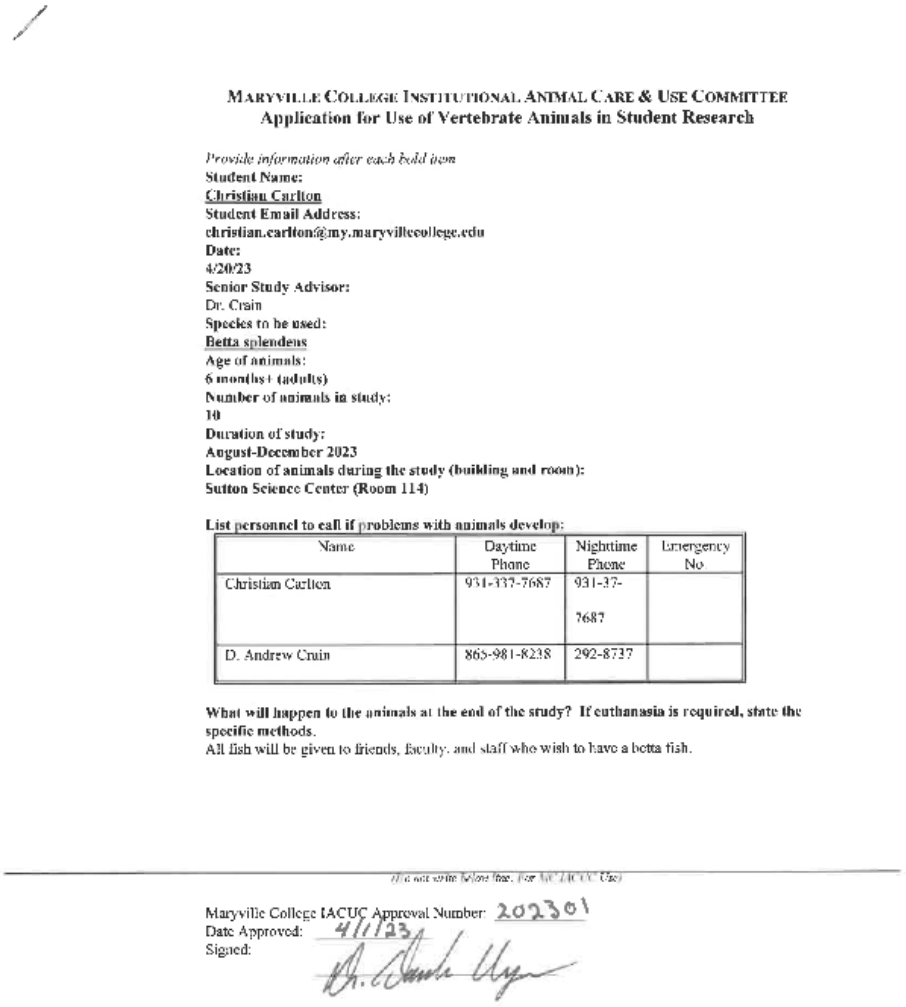
APPENDICES

APPENDIX 1: Example of data collection table.

Table 4: Example of data collection table from Test 1.

Fish #	1	2	3	4	5	6	7	8	9	10
Exposure?	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No
Time (s)										
0-20	FSP	GS	Ct	FSP	FSP	H	FS	GS	Ct	FS
20-40	Ct	GS	FSP	FSH	A	GS	FSH	FSP	S	SS
40-60	Ct	GS	A	FS	GS	GS	FSP	FSP	FSP	SS
60-80	Ct	A	A	FSP	FSH	FSP	FSP	FSP	GS	FS
80-100	Ct	FS	GS	Ct	A	GS	FSH	FSH	FSH	H
100-120	FSP	BR	S	GS	GS	FSP	GS	FSP	FSP	FS
120-140	Ch	BR	FS	Ct	A	FSP	FS	FSP	FSH	Ct
140-160	Ct	BR	FS	FSP	FSH	FSP	FS	FSP	GS	FSH
160-180	A	BR	FS	B	SS	H	H	FSP	FSH	B
180-200	FSP	FS	S	FS	A	FSH	H	FSP	S	S
200-220	A	FS	FSH	FSH	SS	GS	FSP	FSP	FSH	H
220-240	A	FS	Ct	H	FSH	GS	FSP	FSP	FS	FSH
240-260	B	Ct	FSP	FS	FS	FSP	GS	FSP	Ct	FSH
260-280	Ct	A	Ct	FS	SS	FSH	FSP	FSP	FSP	FSP
280-300	A	FSP	Ct	FS	H	S	FSH	FSH	FSP	H
Total Score	14	7	12	7	10	12	10	15	14	6

APPENDIX 2: Signed IUCUC form for use of live animals in this experiment.



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

Provide information after each bold item
Student Name:
Christian Carlton
Student Email Address:
christian.carlton@my.maryvillecollege.edu
Date:
4/20/23
Senior Study Advisor:
Dr. Crain
Species to be used:
Betta splendens
Age of animals:
6 months+ (adults)
Number of animals in study:
10
Duration of study:
August-December 2023
Location of animals during the study (building and room):
Sutton Science Center (Room 114)

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No
Christian Carlton	931-337-7687	931-37-7687	
D. Andrew Crain	865-981-8238	292-8737	

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.
All fish will be given to friends, faculty, and staff who wish to have a betta fish.

Do not write below line. For IACUC Use

Maryville College IACUC Approval Number: 202301
Date Approved: 4/11/23
Signed:

Figure 5: IUCUC form for the experiment.

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