2017

Pharmaceuticals entering Lake Champlain and their combination effects on developing Zebrafish Embryos

Ariana R. Ferraro

Pharmaceuticals entering Lake Champlain and their combination effects on developing Zebrafish Embryos

Ariana Renee Ferraro
University of Vermont
Marsh Life Science Building, Rm 313
Burlington, VT 05405
aferraro@uvm.edu

Abstract

Human-derived pharmaceuticals have been identified entering surface waters in the United States through wastewater effluent. While there is ample literature about what each compound alone does to aquatic life, little is known about the effects aquatic life may experience from the exposure to many different pharmaceuticals present in the environment. More specifically, are the combination of pharmaceuticals in the environment more detrimental to aquatic life than each pharmaceutical alone?

Zebrafish embryos were used to model what aquatic life in Lake Champlain may experience. Vatovec et al (2016) determined that there are 51 environmentally-relevant human derived pharmaceuticals entering Lake Champlain through wastewater effluent. Out of the 51, acetaminophen, carbamazepine, and diphenhydramine were studied. Embryos were exposed to each pharmaceutical for three days at various concentrations and observed for any developmental defects. The sub-lethal concentration of each pharmaceutical was determined and then this specific concentration was used in subsequent combination experiments.

Results indicated that the exposure to all three pharmaceuticals were more detrimental to embryonic development than each pharmaceutical alone. However, when pharmaceuticals were combined in groups of two, one of the groups was more detrimental to embryos than the combination of all three pharmaceuticals.
Although concentrations used in this experiment were much higher than that found in the environment, the implications of this study are still important. It is necessary to determine and demonstrate at what concentration pharmaceuticals are detrimental to aquatic life so that environmental concentrations never reach the concentrations used in the current

experiment. The outcome of this research stands as a warning as to what may occur if nothing is done about pharmaceuticals entering water sources. Future studies should determine combination effects of pharmaceuticals at environmental concentrations.

Keywords

Zebrafish embryo, Toxicity, pharmaceuticals, Lake Champlain

1. Introduction

Recently, there has been an increased effort to understand the extent to which human-derived pharmaceuticals, which are made to elicit a physiological response in humans, animals, and plants, are entering water sources and how these pharmaceuticals may affect our environment (Kolpin et al., 2002). In 2002, Kolpin et al. provided the first nationwide surveillance, which determined pharmaceuticals, hormones, and other organic wastewater contaminants are in fact entering streams in the United States. Pharmaceuticals have been permeating water sources through incorrect disposal of left-over drugs, agricultural run-off (Ferguson, Bernot, Doll, & Lauer, 2013), and excretion of active ingredients or metabolites after use in humans (Grabicova et al., 2014). These active ingredients or metabolites subsequently reach water treatment systems with outdated technology that is not equipped to filter the pharmaceuticals used in healthcare today (Daughton, 2003).

Many studies have been performed to increase understanding of the effects of single acute exposure to a pharmaceutical. One drug that has been studied in particular is the antidepressant fluoxetine because it has been identified in municipal effluents (Brooks et al., 2003). Brooks et al. (2003) found that the environmental concentration of fluoxetine was not acutely toxic to aquatic life and that the lowest measured fluoxetine

effect levels were at an order of magnitude higher than the highest reported municipal effluent concentrations. Brandhof & Montforts (2010) tested acute exposure of carbamazepine (anticonvulsant), diclofenac (anti-inflammatory) and metoprolol (beta blocker for hypertension), and showed that zebrafish embryos had developmental abnormalities such as heart deformation, growth retardation, and scoliosis when exposed to these drugs at higher concentrations than present in the environment.

Concentrations of pharmaceuticals in the environment are generally low and do not surpass drinking water guidelines or aquatic life criteria (Kolpin et al., 2002). However, Koplin et al. (2002) highlights the need for creating guidelines for substances that do not have them. In addition, organisms in the environment are generally not exposed to these pharmaceutical agents in isolation, but in combination. Thus, Koplin et al. (2002) also emphasizes the need for determining any combination effects that these substances may have on aquatic life, which is vital to protect the environment and, ultimately, human health.

Cleuvers (2003) tested the effects of mixtures of pharmaceuticals on aquatic life. The results showed that tests with a combination of two pharmaceuticals revealed stronger effects on *Daphnia manga, Desmodesmus subspicatus, and Lemna minor* in comparison with pharmaceuticals that were tested separately (Cleuvers, 2003). The goal of the current study is to expand upon these findings by examining the combination of three pharmaceuticals previously found in Lake Champlain.

Pharmaceutical Compound	**Environmentally Relevant Concentration**		**Experimental Concentrations**
Carbamazepine (anticonvulsant)	2.2×10^{-12} mg/mL (2.2 ppt) in finished drinking water from Mississippi River (Wang et al., 2011)	4.7×10^{-12} mg/mL (4.7 ppt) in finished drinking water from Missouri River (Wang et al., 2011)	0.05 mg/mL in 1% DMSO 0.1mg/mL in 1% DMSO 0.2 mg/mL in 1% DMSO 0.4 mg/mL in 1% DMSO
Acetaminophen (analgesic)	1.1×10^{-10} mg/mL (110 ppt) in U.S. surface waters (Kolpin et al., 2002)		0.05 mg/mL 0.1 mg/mL 0.2 mg/mL 0.4 mg/mL
Diphenhydramine (DPH) (anti-histamine)	6.0×10^{-6} mg/mL – 7.0×10^{-5} mg/mL (6-70 ppb) in the Colorado River (Kinney, Furlong, Werner, & Cahill, 2006)		0.05 mg/mL 0.1 mg/mL 0.2 mg/mL 0.4 mg/mL

Table 1. Pharmaceuticals that were studied

Vatovec et al. (2016) found that there are 51 pharmaceuticals in 80% or more of wastewater effluent entering Lake Champlain. Of these 51 environmentally relevant pharmaceuticals, three were used in this experiment: carbamazepine, diphenhydramine, and acetaminophen (*Table 1*). The pharmaceuticals do not have the same mode of action and do not have any known interactions. However, it is still important to understand mixture toxicities of drugs that do not interact, as aquatic organisms in the environment are not only exposed to pharmaceuticals with known drug interactions. These three pharmaceuticals were selected as a result of their prevalence in the literature. They have been identified in a variety of water sources in the United States and their effects on aquatic life are beginning to be better understood. When interpreting results, there are ample resources to compare results of the experiment to those in the literature. They were

also selected because of personal interest. In addition, the prices of the pharmaceuticals selected were within our budget.

Carbamazepine is an anticonvulsant and is thought to act by reducing polysynaptic responses and blocking post-tetanic potentiation. The drug also has some analgesic effects. Carbamazepine, or Tegretol, is used to treat seizures due to epilepsy and pain due to trigeminal neuralgia (Novartis Pharmaceuticals Corporation, 2009).

Diphenhydramine (DPH) is an antihistamine with sedative and anticholinergic effects. Diphenhydramine is thought to act by competing with histamines for cell receptor sites on effector cells. DPH, is used to reduce allergic reactions to blood or plasma or during anaphylaxis. It is also used to treat motion sickness and parkinsonism (US Food and Drug Administration, 2013).

Acetaminophen is an analgesic and an antipyretic. It is one of the most widely used medicines in the United States 24.6 billion doses were sold alone in 2008 (Dal Pan, 2009). Acetaminophen is thought to work by inhibiting prostaglandin H synthase (Boutaud et al, 2002). It is found in many prescriptions and in many over the counter medicines used to treat cold and flu symptoms (Dal Pan, 2009).

Zebrafish, *Danio rerio*, served as the model organism for this study. This species has been used as a model organism in developmental biology and molecular genetics and more recently, toxicology. Using zebrafish to study toxicity is valuable because more is known about normal developmental milestones and parameters in zebrafish than any other fish species. This makes it particularly straightforward for studying any abnormalities that may occur due to toxicity exposure (Hill et al., 2005). Researchers have identified and understand morphological, biochemical, and physiological processes

at all stages of early development, adolescent, and both sexes of the adult fish (Hill et al., 2005).

Apart from the in-depth knowledge that is available about normal development in the zebrafish, the species is a great model organism for toxicology studies for many other reasons. Zebrafish are approximately 1-1.5 inches in length. This small size reduces housing needs and husbandry costs compared to other fish used as model organisms such as trout. Since larvae and adults are small less drug is needed to make dosing solutions. This helps to reduce waste accumulation during an experiment. Adult zebrafish have a high fecundity and can often lay 200-300 embryos every five to seven days. Lastly, zebrafish embryos are also transparent. This makes it easy for the observer to notice any phenotypic abnormalities that may result from drug toxicity screening (Hill et al., 2005).

Understanding combination effects of pharmaceuticals on developing embryos is extremely important for a variety of reasons. Pharmaceuticals entering water sources through wastewater effluent have the ability to disrupt the environment. As of right now, many of the pharmaceutical concentrations in water sources are not acutely toxic to aquatic life. However, combination effects must be considered and chronic exposure over multiple generations is of concern. Even if only small organisms are affected by pharmaceutical exposure, the impact of pharmaceuticals entering water sources could be detrimental to local ecosystems and eventually the environment.

In this study we hypothesized that exposure to the combination of pharmaceuticals will be more detrimental to zebrafish embryo development than exposure to a single pharmaceuticals alone. One objective of the experiment was to determine the sub-lethal concentrations of the three drugs that results in developmental

abnormalities. The second objective was to determine the combination effects of the three drugs on developing embryos based upon these sub-lethal concentrations. With these objectives completed, there will be a better understanding of what may actually be happening in the environment, as these human derived pharmaceuticals are not isolated from one another.

2. Methods

The pharmaceuticals (i.e., drugs) that were studied were obtained from Sigma-Aldrich, Carbamazepine (C4024), Acetaminophen (A7085), and Diphenhydramine (D3630). Zebrafish embryos were provided by the Ebert Laboratory and all procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC) protocol #12-055.

Zebrafish embryos were collected by removing a set of adults, which consists of two males and three females, from their tanks and placing the zebrafish in spawning tanks that consist of a lid and an insert that has holes so that the embryos can fall through and be separated from the adult fish. The males and females were then separated in the tank by an insert overnight and released in the morning to spawn in shallow water. The embryos were placed into egg water and subsequently transferred to 24–well dishes for their treatments. Treatments began directly following fertilization to mimic environmental conditions.

To test each drug for lethal and sub-lethal concentrations ten embryos were placed in a well with 1 mL of 100% egg water to act as the control, ten embryos were placed in each well with a drug concentration of 0.05 mg/mL (Treatment 1, T1), 0.1 mg/mL (Treatment 2, T2), 0.2 mg/mL (Treatment 3, T3), and 0.4 mg/mL (Treatment 4, T4). This

method was replicated three times for carbamazepine and diphenhydramine and two times for acetaminophen, using a different batch of embryos in each replication. When testing carbamazepine, the control solution consisted of egg water that is 1% Dimethyl Sulfoxide (DMSO) as carbamazepine is insoluble in water. Each treatment was dissolved in 1% DMSO along with the appropriate drug concentration.

An important breakthrough was discovered when testing with acetaminophen. Instead of placing ten embryos in each well, five embryos were placed in each well. The number was reduced because when testing for developmental abnormalities in embryos exposed to acetaminophen, the embryos were all dead at day two of the experiment. Therefore, the number of embryos in each well was reduced as the embryos may have been dying from hypoxia due to the number of embryos in 1 mL of solution. This new technique was also used when testing for developmental abnormalities in embryos exposed to diphenhydramine and it was used in all combination treatments.

After 24 hours of treatment, embryos were manually dechorionated and were observed daily at 24 hours post fertilization (hpf), 48 hpf, and 72 hpf under a dissecting microscope. Pictures were taken with SPOT software on a Nikon dissecting microscope to document any and all abnormalities of the treatment groups in comparison to the control group. In addition, spontaneous tail movements of the embryos at 24 hpf were studied when testing for developmental abnormalities that may be associated with diphenhydramine exposure because the drug has some sedative effects. To determine if spontaneous movement was affected, each of the control embryos and each of the embryo undergoing diphenhydramine treatments were observed under a microscope for one minute at day one (18-26 hours post-fertilization) (Huang et al., 2010). The number of

times each embryo bends its tail was then recorded. At 72 hpf, eye size was noticeably smaller for embryos exposed to 0.4 mg/mL of acetaminophen. Therefore, SPOT software was used to determine the area (μm^2) of each eye from photographs.

Sub-lethal concentrations of each separate drug were established at 72 hpf, based upon which treatment concentration exhibited the most phenotypic abnormalities without killing all embryos. These concentrations were then combined into 1 mL of egg water containing 1% DMSO. There was a control well of 1% DMSO in egg water with five embryos, and a treatment well of the mixture and five embryos. This treatment was replicated once as only one clutch of embryos was used. Again, after 24 hours of treatment, embryos were dechorionated and were observed at 24 hpf, 48 hpf, and 72 hpf using a dissecting microscope. Developmental abnormalities were recorded using SPOT. Spontaneous tail movements were recorded for day one of the experiment (18-28 hpf) and eye size was recorded at 72 hpf.

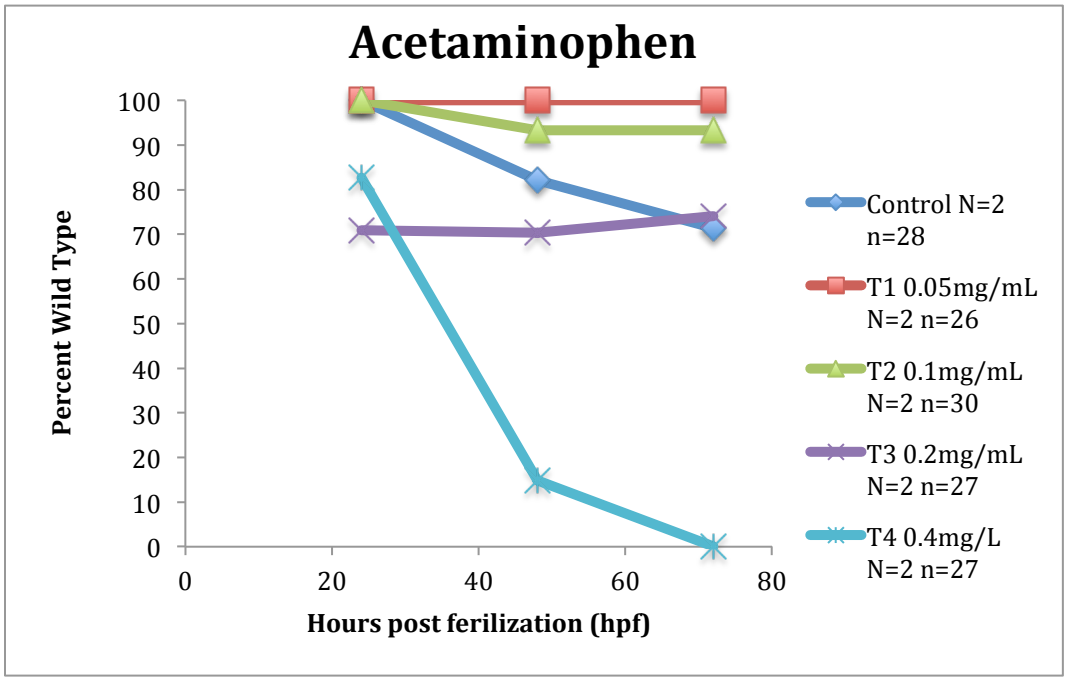
After the combination effects of all three pharmaceuticals were observed and documented, a treatment with the sub-lethal concentrations of diphenhydramine and acetaminophen was conducted. This treatment was replicated twice. A treatment with the sub-lethal concentrations of diphenhydramine and carbamazepine and a treatment with the sub-lethal concentrations of acetaminophen and carbamazepine were also conducted. These treatments were each replicated once. Likewise, after 24 hours of treatment, embryos were manually dechorionated and observed at 24 hpf, 48 hpf, and 72 hpf using a dissecting microscope. SPOT software was used to photograph any developmental abnormalities seen. Spontaneous tail movements were recorded at day one of the

experiment for any treatment that contained diphenhydramine. Eye size was recorded at 72 hpf for any treatment that contained acetaminophen.

Results were organized into line graphs and tables. Percent wild-type over 72 hours was displayed in a line graph for each drug and drug combinations. In tables, results were organized into percent wild-type, percent mild (one abnormality), percent moderate (two abnormalities), percent serious (three abnormalities), percent severe (4 or more abnormalities), and percent dead. Phenotypic abnormalities were further organized by reporting percent of specific phenotypes seen at 72 hpf, such as edema, tail deformities, spine deformities, etc, for sub-lethal concentrations of each drug and subsequent combination treatments. An unpaired *t*-test was used to determine differences in eye size from T4 (0.4mg/mL) acetaminophen treatment. A multiple comparisons one way ANOVA was used to compare eye size in T4 of acetaminophen versus combination treatments that included acetaminophen. A multiple comparisons one way ANOVA was used to compare the frequency of tail bends across the controls and all treatments of diphenhydramine. Again, a multiple comparisons one way ANOVA was then used to compare the diphenhydramine treatment concentration that was eventually chosen for further combination testing against combination treatments that included diphenhydramine.

3. Results

Acetaminophen results:



Graph 1: Percent of wild type zebrafish embryos over time exposed to four different treatments of acetaminophen.

Phenotypic Categories in Acetaminophen						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
Controls						
24	100	0	0	0	0	0
48	82.14	3.57	14.28	0	0	0
72	71.43	10.71	3.57	0	0	14.29
T1 (0.05mg/mL)						
24	100	0	0	0	0	0
48	100	0	0	0	0	0
72	100	0	0	0	0	0
T2 (0.1mg/mL)						
24	100	0	0	0	0	0
48	93.33	6.66	0	0	0	0
72	93.33	6.66	0	0	0	0
T3 (0.2mg/mL)						
24	70.96	29.03	0	0	0	0

48	70.37	29.63	0	0	0	0
72	74.07	25.93	0	0	0	0
T4 (0.4mg/mL)						
24	82.76	17.24	0	0	0	0
48	14.81	77.78	7.41	0	0	0
72	0	59.26	14.81	25.93	0	0

Table 1: Percent of wild type zebrafish embryos in different acetaminophen treatments over the course of three days.

At 24 hours post fertilization (hpf) the control group, T1, and T2 had 100% wild type zebrafish embryos. There were no developmental abnormalities present. However, at 24 hpf for T3 and T4, the percent wild-type was 70.96% and 82.76% respectively (*Table 1*). As of day one, T3 and T4 had the most detrimental effects on zebrafish development with 29.03% of embryos with mild phenotypes and 17.24% of embryos with mild phenotypes, respectively. At 48 hpf the percent wild type of the control group decreased while the percent wild type in T1 remained constant. The percent wild-type decreased in T2 and only slightly in T3. However, the percent wild type drastically decreased in T4. At 72 hpf the percent wild type of T1 and T2 remained constant, but the control group decreased in percent wild type and 14.29% of the embryos in the control group were dead. The percent wild type in T3 increased slightly while the percent wild type in T4 dropped to 0% (*Table 1)*. Overall, percent wild type remained fairly steady over the course of three days in the control, T1, T2, and T3 groups. T4 offered the most drastic decrease in percent wild type by day three (See *Graph 1)*, thus the concentration in T4 (0.4mg/mL) was used in subsequent combination experiments.

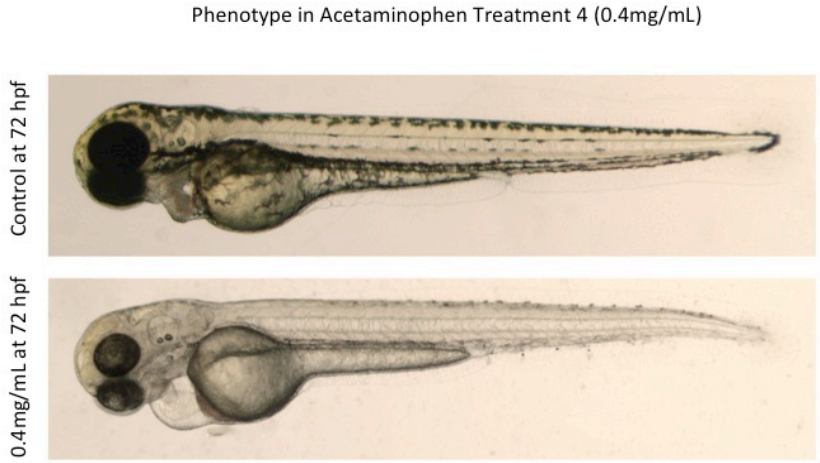
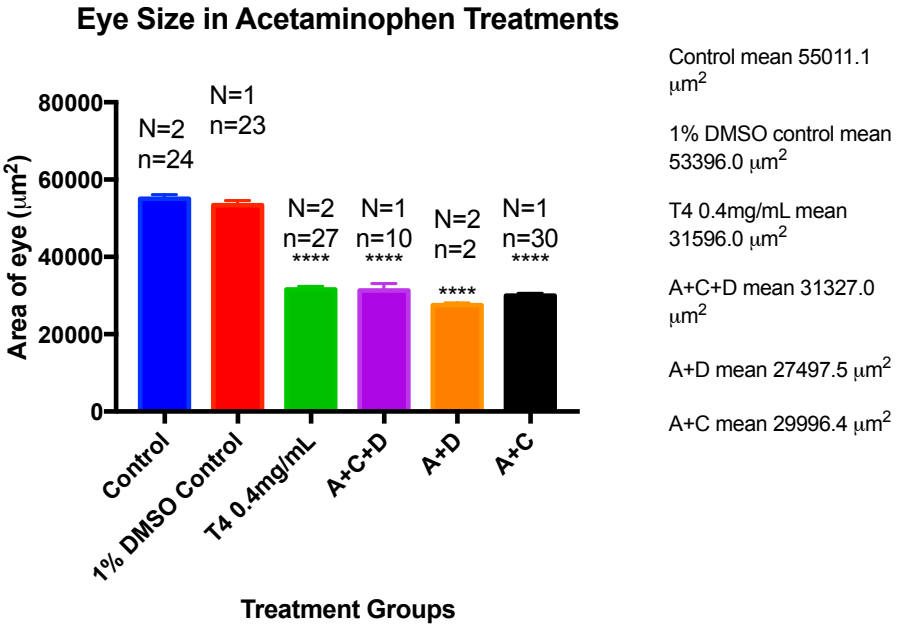


Figure 1: Image of the most common phenotype seen (small eyes) in T4 of acetaminophen at 72 hpf.

In T4 at 72 hpf 85.19% of embryos had small eyes, 40.74% had edema, and 40.74% were less pigmented. *Figure 1* exhibits all three phenotypes.

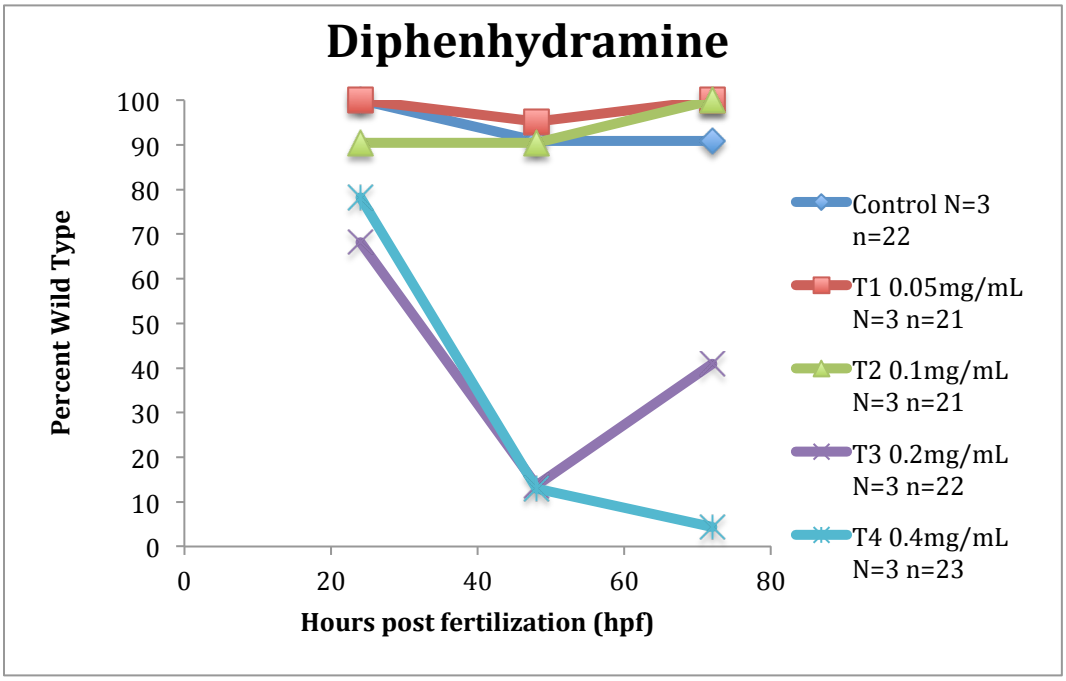


Graph 2: Eye size differences at 72 hpf between controls and various treatments that included acetaminophen. Graph shows standard error of the mean bars. ANOVA multiple comparisons.

At 72 hpf eye size between the control and treatment 4 (0.4 mg/mL) embryos were noticeably different. Their size was measured and an unpaired t test was conducted,

which showed that zebrafish embryo eyes were significantly smaller (P <0.0001) in treatment four than in the control group. A one-way ANOVA with multiple comparisons was then conducted on eye size data from the control, 1% DMSO control, T4, A+C+D, A+D, and A+C groups. Embryo eyes were significantly smaller in treatment 4 than in the 1% DMSO control group (P value <0.0001, *Graph 2)*. Differences in eye size were not significant between the control group and the 1% DMSO control. In addition, differences in eye size were not significant between T4 and A+C+D, T4 and A+D, T4 and A+C, A+C+D and A+D, A+C+D and A+C, and A+D and A+C (*Graph 2*).

Diphenhydramine results:



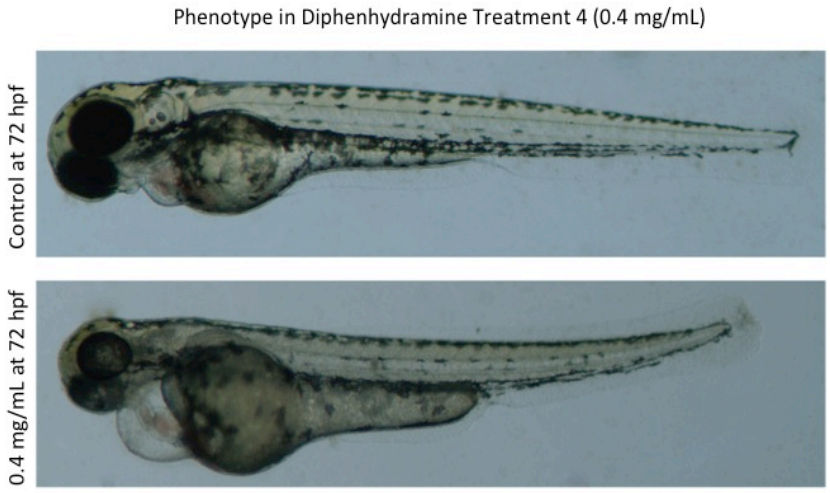
Graph 3: Percent of wild type zebrafish embryos over time exposed to four different treatments of diphenhydramine.

Phenotypic Categories in Diphenhydramine						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
Controls						
24	100	0	0	0	0	0
48	90.48	9.09	0	0	0	0
72	90.9	9.09	0	0	0	0
T1 (0.05mg/mL)						
24	100	0	0	0	0	0
48	95.24	4.76	0	0	0	0
72	100	0	0	0	0	0
T2 (0.1mg/mL)						
24	90.48	9.52	0	0	0	0
48	90.48	9.52	0	0	0	0
72	100	0	0	0	0	0
T3 (0.2mg/mL)						
24	68.18	31.81	0	0	0	0
48	13.63	81.82	0	0	0	0
72	40.91	45.45	0	0	0	13.63
T4 (0.4mg/mL)						
24	78.26	21.74	0	0	0	0
48	13.04	82.61	4.35	0	0	0
72	4.35	73.91	4.35	0	0	17.39

Table 2: Percent of wild type zebrafish embryos in different diphenhydramine treatments over the course of three days.

At 24 hpf the control and T1 were both 100 percent wild type. T3 had the lowest percent wild type at 68.18%, T4 had the second lowest at 78.26% and T2 had the third lowest at 90.48%. At 48 hpf all groups decreased in percent wild type except T2. T3 and T4 had comparable percent wild type at 13.63% and 13.04%, respectively. At 72 hpf the percent wild type in the control group remained the same. The percent wild type increased in T1, T2, and T3. In T4 the percent wild type decreased to 4.35%. By day

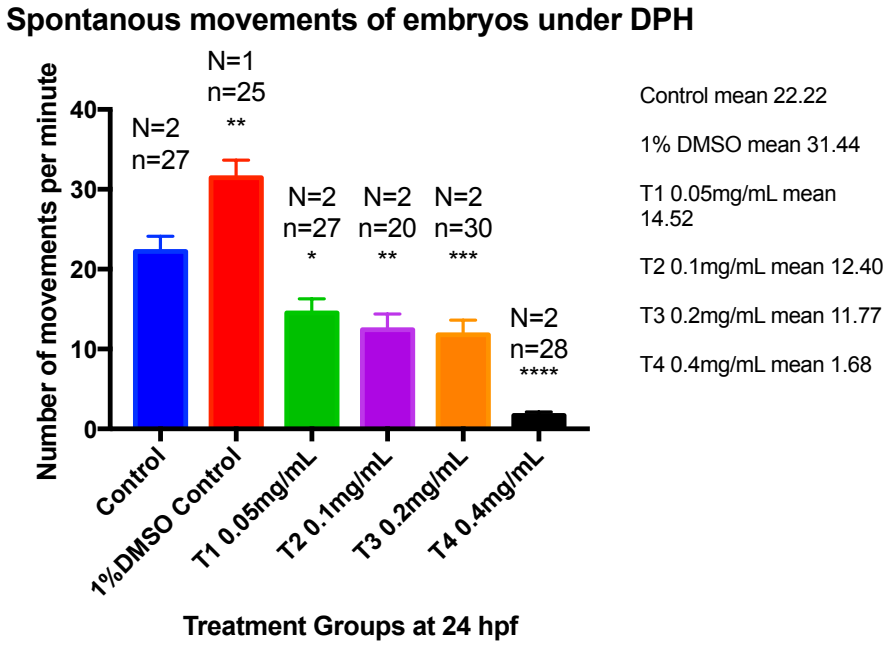
three of the experiment (72 hpf), T4 has the lowest percent wild type while T1 and T2 had the highest at 100% (*Table 2)*. In addition, T4 had the most drastic decrease in percent wild type while T1 and T2 stayed fairly stable (*Graph 3).* T4 had the highest percent mild, moderate and dead at 73.91%, 4.35%, and 17.39%, respectively. Therefore,



treatment 4 concentration (0.4mg/mL) was used in subsequent combination experiments.

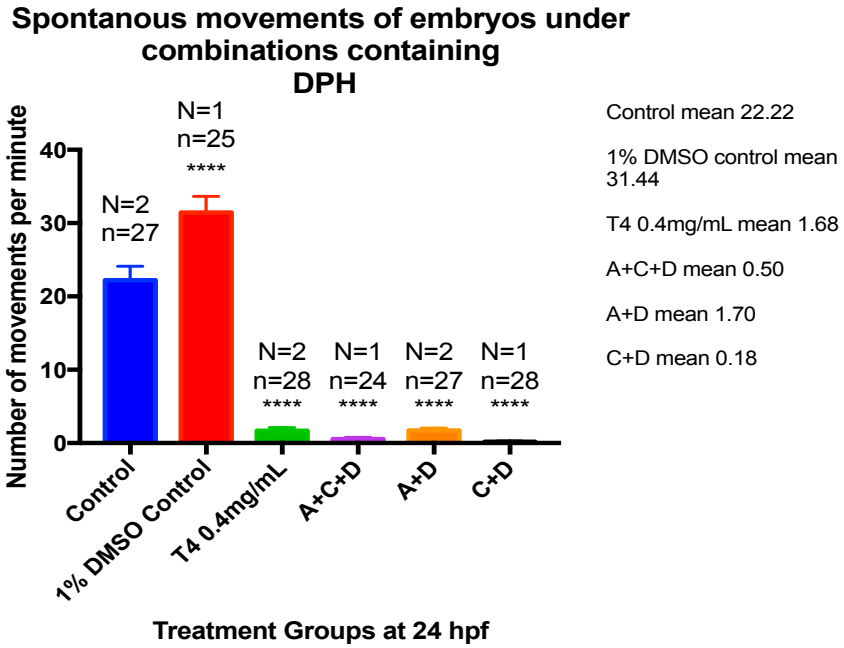
Figure 2: Image of the most common phenotype seen (edema) in T4 of diphenhydramine at 72 hpf.

At 72 hpf in T4 the embryos had the highest percent of edema at 69.57% and the highest percent of tail deformities at 8.70%. In addition, 4.35% of embryos in T4 at 72 hpf had spine deformities. *Figure 2* shows only an edema.



Graph 4: Number of movements per minute in each DPH treatment group at 24 hpf. Graph shows standard error of the mean bars. ANOVA multiple comparisons.

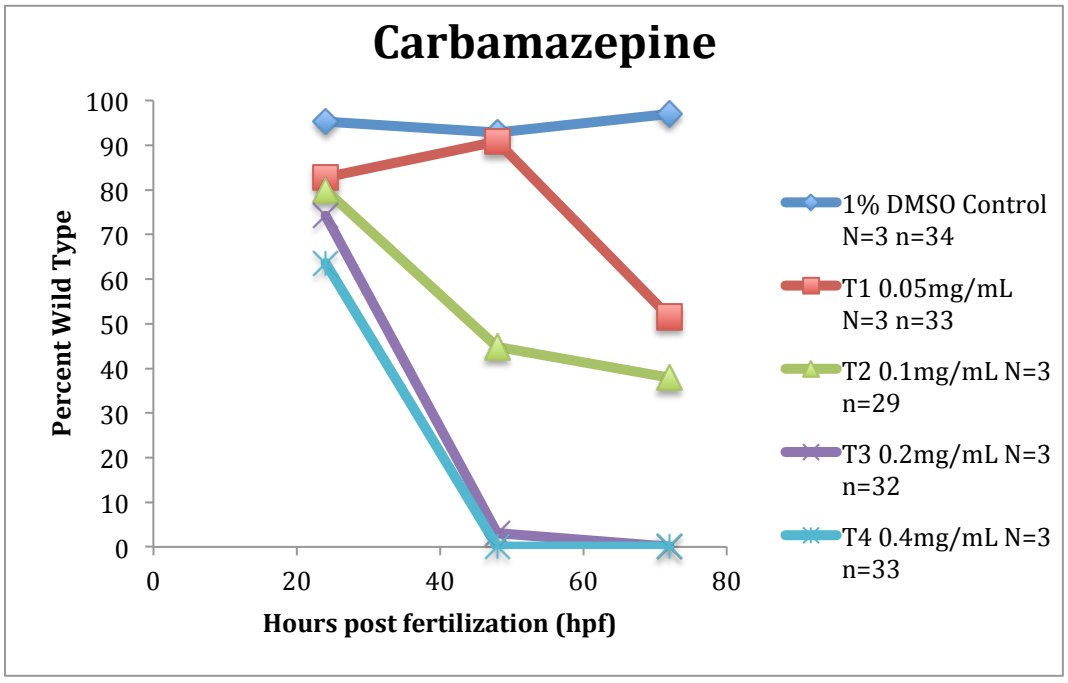
Spontaneous movements were recorded for each treatment group to better characterize the effect DPH exposure may have had on developing zebrafish embryos. An one way ANOVA multiple comparisons test was conducted. The difference in number of movements per minute was significantly different in the control group compared to the 1% DMSO control. Treatments 1, 2, and 3 all had significantly less movement than in the control group. The number of movements per minute was most significantly different in the control group compared to T4. Treatments 1, 2, 3, 4 all had significantly less movement than in the 1% DMSO control group. The difference in movement between T4 and treatments one, two, and three, was also significant. The difference in number of movements between T1, T2, and T3 were not significant (See *Graph 4)*.



Graph 5: Number of movements per minute at 24 hpf in each combination group containing DPH compared to T4. ANOVA multiple comparisons. Graph shows standard error of the mean bars.

Differences between T4 and the combinations of A+C+D, A+D, and C+D were not significant. In addition, differences in tail movements were also not significant between A+C+D and A+D, A+C+D and C+D, and A+D and C+D (See *Graph 5)*.

Carbamazepine results:



Graph 6: Percent of wild type zebrafish embryos over time exposed to four different treatments of carbamazepine.

Phenotypic Categories in Carbamazepine						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
1% DMSO control						
24	100	0	0	0	0	0
48	95.24	4.76	0	0	0	0
72	97.62	2.38	0	0	0	0
T1 (0.05mg/mL)						

24	82.86	5.71	11.43	0	0	0
48	90.91	9.09	0	0	0	0
72	51.52	33.33	6.06	0	0	9.09
T2 (0.1mg/mL)						
24	83.33	16.67	0	0	0	0
48	44.83	41.38	3.45	0	3.45	6.90
72	37.93	20.69	3.45	0	0	37.93
T3 (0.2mg/mL)						
24	79.41	8.82	5.88	0	5.88	0
48	3.23	77.42	12.90	0	0	6.45
72	0	38.71	25.81	0	0	35.48
T4 (0.4mg/mL)						
24	65.63	25	0	0	9.38	0
48	0	22.58	61.29	0	9.68	6.45
72	0	10.34	31.03	6.90	3.45	48.28

Table 3: Percent of wild type zebrafish embryos in different carbamazepine treatments over the course of three days.

At 24 hpf the controls had 100% wild type. T1 and T2 were comparable at 82.86% wild type and 83.33% wild type, respectively. T3 had the second lowest percent wild type of 79.41 at 24 hpf, while T4 had the lowest at 65.63%. At 48 hpf the controls decreased to 95.24% wild type, while T1 had increased to 90.91% wild type. T2, T3, and T4, all plummeted in percent wild type to 44.83%, 3.23%, and 0%, respectively. By 72 hpf controls were at 97.62% wild type. T1 had 51.52% wild type and T2 had an even smaller percent wild type at 37.93%. Both T3 and T4 had 0% wild type at 72 hpf (*Table 3)*.

T2 was picked for the concentration used in subsequent combination experiments. Both T3 and T4 had 0% wild type at 72 hpf, but these treatments had a high percent of death at 35.48% and 48.28% respectively. Although T2 did have a higher percent of death at 37.93% compared to T3, T2 was picked for subsequent experiments before a proper sample size was reached due to time constraints. In the end, more trials were done to reach an appropriate sample size, which revealed that T3 should have been the concentration used in subsequent combination experiments (*Table 3)*.

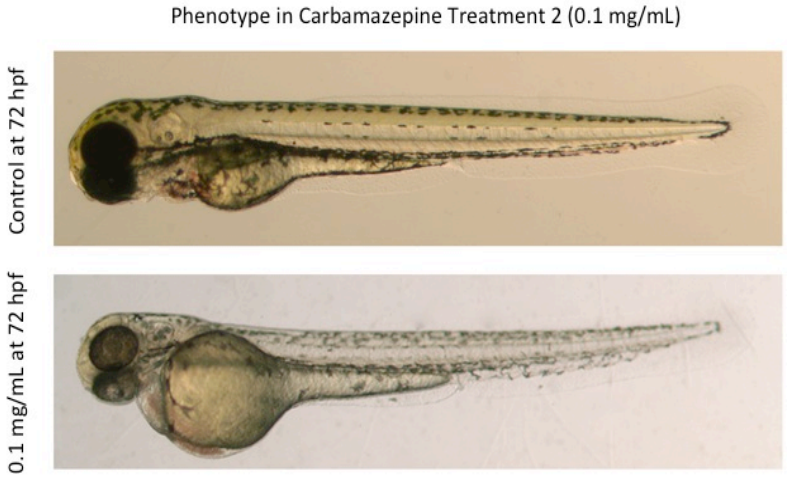
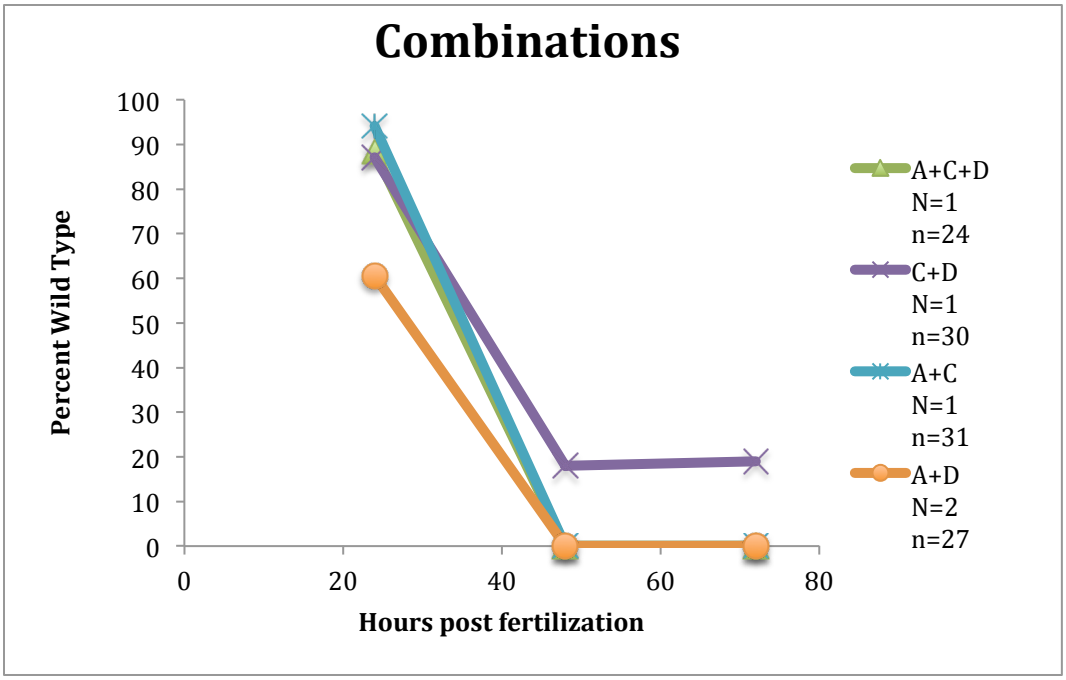


Figure 3: Image of the most common phenotype seen (edema) in T2 of carbamazepine at 72 hpf.

At 72 hpf in T2 17.24% of embryos had edema. This was the most common phenotype seen in this treatment group. In addition to edema, 3.45% of embryos had tail deformities, spine deformities and were less pigmented. *Figure 2* shows an edema.

Combination results:



Graph 7: Percent of wild type zebrafish embryos over time exposed to different combination treatments.

Phenotype categories seen in A+C+D						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
24	88.46	11.54	0.00	0.00	0.00	0.00
48	0.00	0.00	41.67	58.33	0.00	0.00
72	0.00	0.00	41.67	0.00	0.00	58.33
Phenotype categories seen in A+D						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
24	60.61	36.36	3.03	0.00	0.00	0.00
48	0.00	0.00	22.22	18.52	25.93	33.33
72	0.00	0.00	7.41	0.00	0.00	92.59
Phenotype categories seen in A+C						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead
24	92.12	2.94	0.00	0.00	0.00	0.00
48	0.00	0.00	32.36	58.06	6.45	3.23
72	0.00	0.00	0.00	0.00	96.77	3.23
Phenotype categories seen in C+D						
hpf	%WT	%mild	%moderate	%serious	%severe	%dead

24	87.10	12.90	0.00	0.00	0.00	0.00
48	23.33	40.00	20.00	13.33	0.00	0.00
72	13.33	30.00	10.00	13.33	3.33	30.00

Table 4: Percent of wild type zebrafish embryos in different combination treatments over the course of three days.

At 24 hpf the combination treatment with acetaminophen and diphenhydramine (A+D) had the lowest percent wild type at 60.60%. The combination treatment with acetaminophen, carbamazepine, and diphenhydramine (A+C +D) and the combination treatment with carbamazepine and diphenhydramine (C+D) had comparable percent wild types at 88.46% and 87.10%, respectively. The combination treatment with acetaminophen and carbamazepine (A+C) had the highest percent wild type at 92.12%. At 48 hpf combination treatments A+C+D, A+C, and A+D all had percent wild types of zero. C+D treatments decreased to 23.33% wild type. At 72 hpf combination treatments A+C+D, A+C, and A+D remained at percent wild types of zero while C+D treatments decreased even more to 13.33% (*Table 4*). The combination of A+D was the most lethal, as 92.59% of embryos were dead by 72 hpf. The second most lethal treatment was the combination of A+C+D as 58.33% of embryos were dead at 72 hpf. The third most lethal treatment was C+D as 30.00% of the embryos were dead at 72 hpf. However, it's important to keep in mind that the C+D treatment still had a wild type percent of 13.33% at 72 hpf while all other combination treatments had 0%. Lastly, only 3.23% of embryos were dead by 72 hpf in A+C treatments (*Table 4).*

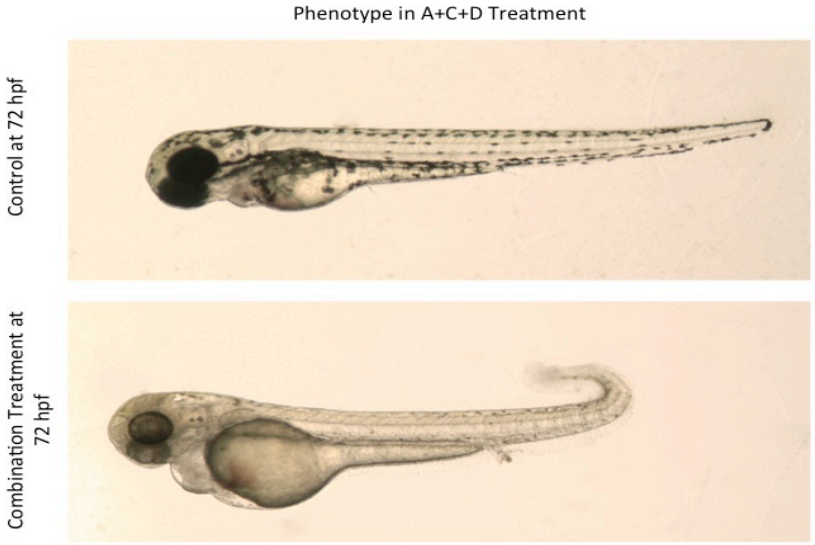


Figure 4: Image of the most common phenotype seen (edema and tail deformities) in 0.4mg of acetaminophen, 0.4mg of diphenhydramine, and 0.1mg of carbamazepine in 1 mL of water.

At 72 hpf 41.67% of the treated embryos had edema and 41.67% of the treated embryos had tail deformities. *Figure 4* shows both abnormalities.

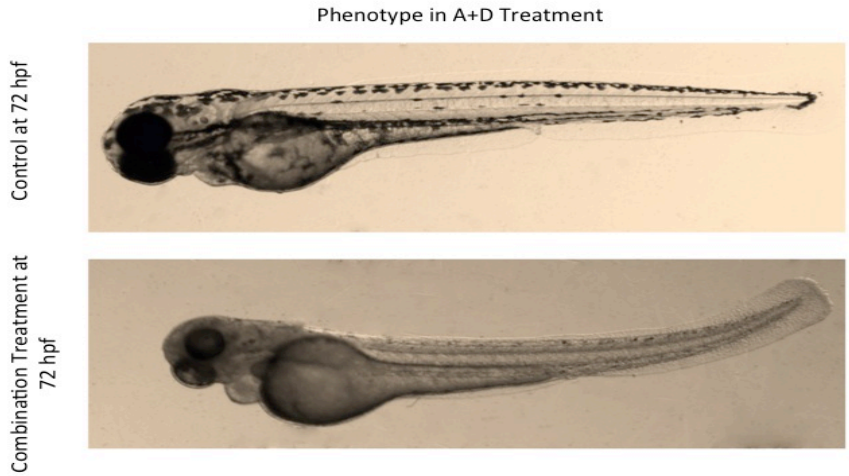


Figure 5:Image of most common phenotype seen in 0.4 mg of acetaminophen and 0.4 mg diphenhydramine in 1 mL of water.

At 72 hpf, 7.41% of embryos had edema and 7.41% of embryos were less pigmented. *Figure 5* shows both abnormalities.

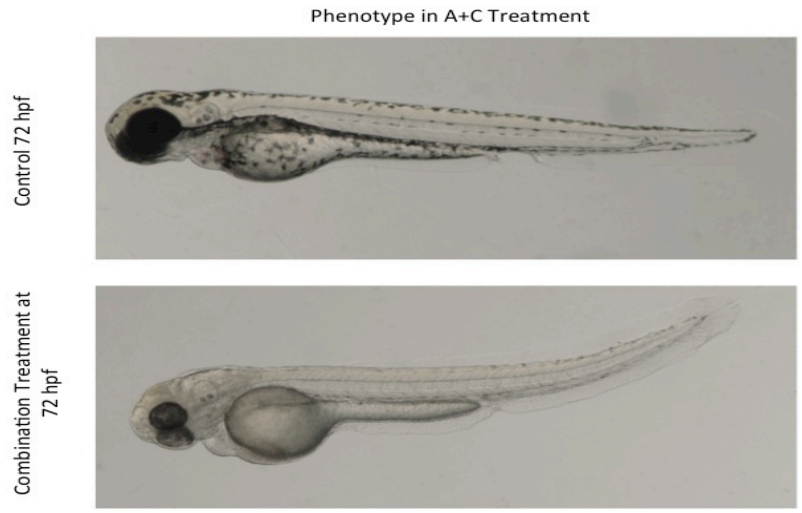


Figure 6: Image of the most common phenotypes seen in 0.4mg of acetaminophen, and 0.1mg of carbamazepine in 1 mL of water.

At 72 hpf, 93.55% of embryos had edema. 93.55% of embryos had small eyes, 93.55% had less pigment, and 93.55% had tail deformities. 3.23% of embryos were extremely deformed. *Figure 6* shows an edema, small eyes, less pigment, and a tail deformity.

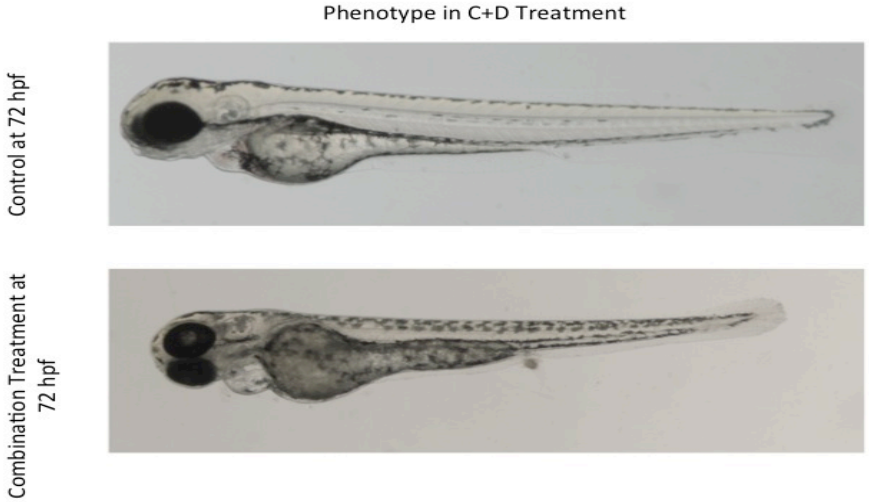


Figure 7: Image of the most common phenotype seen (edema) in 0.4mg/mL of diphenhydramine and 0.1mg/mL of carbamazepine.

At 72 hpf, 53.33% of embryos had edema. 23.33% of embryos had tail deformities. 13.33% of embryos had small eyes and 3.33% of embryos were extremely deformed. *Figure 7* shows an edema.

4. Discussion

Out of all the individual pharmaceuticals tested, carbamazepine had the most detrimental effects on zebrafish embryo development. After 72 hours exposed to T3 (0.2mg/mL) 35.48% of embryos were dead and there were no wild type phenotypes. After 72 hours of exposure to 0.2 mg/mL of diphenhydramine only 13.63% of embryos were dead and 40.91% percent of embryos remained wild type. Embryos treated in 0.2mg/mL of acetaminophen after 72 hours remained alive, with a 74.07% percent wild type. Embryos exposed to 0.4 mg/mL of carbamazepine after 72 hours fared even worse with 48.28% dead and 0% wild types. This was far worse than effects seen at 72-hour exposure to 0.4mg/mL of diphenhydramine (0% wild type and 17.39% dead) or 0.4mg/mL of acetaminophen (0% wild type and 0% dead). With only this information it is appropriate to assume that acetaminophen had the least amount of detrimental effects of embryo development; however, combination trials reveal otherwise.

The combination of 0.4mg/mL of acetaminophen, 0.4 mg/mL of diphenhydramine, and 0.1 mg/mL carbamazepine in 1 mL of water proved to be more detrimental to development than each of the drug concentrations on their own. After 72 hours, there were no wild type embryos and 58.33% of the embryos were dead. In addition, all other combinations tested were more detrimental to development than each drug alone.

Surprisingly, the combination with all three pharmaceuticals was not the most detrimental drug combination. When 0.4mg of acetaminophen and 0.4mg of diphenhydramine were combined 92.59% of embryos were dead at 72 hpf and there were

no embryos without any abnormalities. This result was worse than any results that were seen with each separate drug in this combination.

Yet, other combination experiments provided conflicting results. A mixture of 0.1 mg carbamazepine and 0.4mg diphenhydramine had 13.33% wild type and 30% dead at 72 hpf and a mixture of 0.4mg of acetaminophen and 0.1mg of carbamazepine had 3.23% of embryos dead at 72 hpf with 0% wild type. The mixture with acetaminophen and carbamazepine was not as lethal as 0.1mg of carbamazepine alone as that treatment yielded 37.93% wild type and 37.93% dead, but the A+C mixture had 0% wild type embryos. However, the A+C mixture was more detrimental than 0.4mg of acetaminophen alone. The C+D mixture had a smaller percent wild type than carbamazepine but not diphenhydramine, and was more lethal than diphenhydramine alone and almost as lethal as 0.1mg carbamazepine alone.

One possible explanation as to why A+C and C+D had a lower percent of death and a higher percent of wild type embryos is that these treatments only contained 0.5mg of total drugs added in 1 mL compared to 0.8mg of total drugs in 1 mL of water in A+D treatments. Unfortunately, this does not explain why A+D treatments had worse effects on embryos than A+C+D treatments that contained a total of 0.9mg drug in 1mL. In addition, A+D treatment did not include carbamazepine, which showed to be the most lethal drug out of all three pharmaceuticals. Why is it that the only combination without carbamazepine was the most lethal? None of the drugs tested have known interactions so it would be unlikely that one combination with two drugs be worse than the others. It will be important in future studies to increase the replicate size in order to better understand combination effects. In this study water quality was not checked throughout the

experiments. For example, it may be possible that the A+D mixture had an unfavorable pH for developing zebrafish embryos and that is why the A+D mixture was so toxic. Zebrafish require a pH between 6-8 (Sanders, 2009) and the adult zebrafish in our lab are kept at a pH of ~7.81. Monitoring water quality through pH should be an important part of future studies.

It is difficult to say whether acetaminophen or diphenhydramine had more negative effects on embryo development due to the conflicting results seen in combination treatments. More research should be done to further tease out which single pharmaceuticals and combinations are most detrimental to zebrafish embryo development. Future studies should test pharmaceuticals together at the same concentrations and then with every possible combination of drug and concentration to get a more complete picture.

As for phenotypic results concerning exposure to a single pharmaceutical, lack of pigmentation seen in embryos treated with acetaminophen at 0.4 mg/mL is consistent with David & Pancharatna (2009) where embryos exposed to acetaminophen concentrations of 5.0×10^{-5} mg/mL and 1.0×10^{-4} mg/mL exhibited lack of pigmentation. However, lack of pigmentation was not observed in embryos treated with an acetaminophen concentration of 1.0×10^{-5} mg/mL (David & Pancharatna, 2009). David & Pancharatna (2009) also observed deformed tails, abnormal swimming patterns and lack of response to external stimuli in all treatment groups. However, my research showed that embryos exposed to 0.4 mg/mL of acetaminophen had no deformed tails, while swimming patterns and lack of response to external stimuli were not examined. Although significantly smaller eyes were seen in embryos treated with 0.4 mg/mL

acetaminophen, no observations of this phenotype were made in David & Pancharatna (2009). More research needs be done to determine if smaller eyes are a consistent phenotype seen in embryos exposed to high acetaminophen concentrations.

Selderslaghs, Blust & Witters (2012), treated zebrafish embryos with diphenhydramine at a concentration range of $2.55x10^{-3}$ mg/mL to $9.96x10^{-2}$mg/mL and found abnormalities in heartbeat, circulation, skeletal structure and swimming behaviors at 144 hours post treatment. In my research I found that the most common abnormality in embryos exposed only to 0.4mg/mL of DPH was the presence of edema, the second most common was tail deformities and the third most common was spinal deformities. Heartbeat, circulation, and swimming behavior were not recorded in my experiment. However, embryos did show significantly less spontaneous tail movement at 24hpf when exposed to diphenhydramine. While this spontaneous movement is not a swimming behavior present at 144 hpf, the results of this experiment are supported by Selderslaghs, Blust & Witters's (2012) findings that diphenhydramine, which is known to have sedative effects, alters swimming behavior. In addition, deformities in the tail and spine are supported by skeletal deformities observed in embryos in the Selderslaghs, Blust & Witter's study (2012).

Brandhof & Montforts (2010) treated embryos with carbamazepine at concentrations of 0.05mg/mL, 0.1 mg/mL, and 0.2 mg/mL and saw tail deformities at 0.05 mg/mL, tail deformities and growth retardation at 0.1 mg/mL, and tail deformities, heart deformities, and growth retardation at 0.2 mg/mL. In my research, embryos exposed to 0.1 mg/mL of carbamazepine also had tail deformities. However edemas, spine deformities, and embryos with less pigment were observed while they were absent in the

Brandhof & Montforts (2010) study. Lastly, carbamazepine exposure in my experiment seemed to cause more embryos to die by 72 hpf at 0.1mg/mL (37.93%) compared to the number of embryos that were dead by 72 hpf at 0.1 mg/mL (0%) in Brandhof & Montforts (2010) study. This discrepancy may be a result of the exposure to both carbamazepine and DMSO, as Brandhof & Montforts used an ultrasonic bath and stirring to dissolve carbamazepine. Or this discrepancy may be a result of Brandhof & Montforts only using eggs from a single clutch. This experiment performed replicates among different clutches, which is important because there can be a "large variability between batches of eggs in their sensitivity to toxicants (Brandhof & Montforts, 2010)."

There are a few limitations to take into consideration while interpreting results of the study. Carbamazepine has been found in surface waters, but since carbamazepine is not very soluble in water the drug is also found in sediment (Oetken, Nentwig, LÖffler, Ternes, & Oehlmann, 2005). Zebrafish embryos in this study were exposed to carbamazepine in water not sediment, so the drug had to be dissolved in DMSO. As a result, embryos were also exposed to DMSO. However, not the entire drug dissolved in the 1% DMSO solution and the amount of DMSO in the solution could not be increased without harming the embryos. Therefore, results concerning exposure to carbamazepine may be inconsistent due to the inability of the drug to fully dissolve in solution. Future studies may instead fine-tune the amount of DMSO and carbamazepine to get a more accurate idea of what this drug can do to developing zebrafish embryos. Future studies could also use Brandhof & Montforts (2010) method, which did not utilize DMSO at all, for dissolving carbamazepine.

Second, the methods used made it impossible for embryos to be exposed to pharmaceuticals from the moment they were fertilized up until 72 hpf. Often, 30 minutes to 45 minutes would pass before all embryos were all transferred from the spawning tanks to their treatment dishes. To better mimic what would be happening in the environment, it may be of interest to prepare the drug solution to be used in spawning water for future studies.

Another limitation was inherent in the methodology to collect diphenhydramine spontaneous movement data. After all the embryos were dechorinated, their spontaneous movement was measured by recording the amount of times each embryo moved its tail in one minute. The controls were always measured first, then treatment 1, treatment 2, treatment 3, and lastly treatment 4. In combination experiments where there was only one treatment, the control embryo's movements were recorded first, and then the treatment embryo's movements were measured. Results indicated that treatment groups had significantly less spontaneous tail movements. However, was this a result of the order that the data was collected in? Did the treatment groups have more time to grow accustomed to the shock of being dechorionated and thus move less? Did the treatment groups move less because more time had passed by the time their movements were recorded compared to the treatment groups? It is important to keep in mind that spontaneous movement behavior is only present in embryos 18-26 hpf, and it may be that movement is different at 18 hpf compared to 19 hpf. To isolate the effects of diphenhydramine from the effects time may have had on spontaneous movement it would be beneficial to record movement in a video and then record the data in order to ensure embryos are being compared at the same moment time. Lastly, spontaneous movement

data collected at 24 hpf for the control, T1, T2, T3, and T4 was not from the same clutch of embryos that provided phenotypic data. This was because embryos that provided data for spontaneous movement at 24 hpf were dead on day two of the experiment so their phenotypes were not recorded. Since DPH experiments where phenotypes were recorded did not have all embryos dead by day two, it can be assumed that the clutch of eggs used to record movement data were not healthy. This lack of health may have contributed to less movement overall for all treatment groups. Although combination results indicated that exposure to more pharmaceuticals did not significantly decrease spontaneous tail movements, this may not be true especially if the embryos in the T4 group were not healthy to begin with. In the future it would be best to use the same clutch of eggs for spontaneous movement data and phenotypic data.

While collecting eye size data for embryos treated with acetaminophen and diphenhydramine the majority of embryos were dead by day three. This left a sample size of 2. More trials should be done to increase the sample size in order to properly compare eye size across all experiments containing acetaminophen.

Phenotypic results and categories were completely subjective. As with anything that is subjective there is room for error and discrepancies. Future studies may want to solely research quantitative aspects of zebrafish development such as heartbeat, eye size, spontaneous movement, etc.

Lastly, the concentrations that were studied are much higher than what is actually in surface waters in the United States. However, the concentrations in the environment will continue to rise. In the environment carbamazepine has been reported as having concentrations up to 2.2×10^{-12} mg/mL. The concentration used in combination treatments

(0.1mg/mL) is $4.54x10^{10}$ times higher than what is found in the environment. Acetaminophen combination treatments used a concentration of 0.4mg/mL, which is $3.63x10^{9}$ times higher than what is found in the environment ($1.1x10^{-10}$ mg/mL). Lastly, diphenhydramine concentrations (0.4 mg/mL) used in combination treatments are $6.66x10^{4}$ times higher than what is found in the environment ($6.0x10^{-6}$ mg/mL).

While this is somewhat encouraging because these phenotypic effects most likely will not be seen in the environment, it is still of extreme importance to understand exactly what is happening in the environment. Future studies should test drugs in combination at their environmental concentrations. It would also be beneficial to grow a clutch of zebrafish embryos in conditions that are equal to drug concentrations in the environment to study any effects the combination of drugs may have on future generations. In addition, only three out of the 51 pharmaceuticals found entering Lake Champlain were studied in this experiment. Therefore, it is important to determine how the remaining 48 pharmaceuticals in the lake may be affecting aquatic development alone and in combination. These studies must be done in order to provide a necessary wake up call to update wastewater treatment systems before pharmaceuticals in effluent becomes too toxic and to protect our environment.

5. Conclusions

Overall, the goals of this experiment were reached. Sub-lethal concentrations were determined for acetaminophen, diphenhydramine, and carbamazepine. Once combined, the mixture of the three pharmaceuticals proved to be more detrimental to developing zebrafish embryos than each of the pharmaceuticals alone. However, when pharmaceuticals were combined in groups of two the results ended up being quite

conflicting as the acetaminophen and diphenhydramine combination was more detrimental than the combination of the three pharmaceuticals. Since replicates in combination experiments were low and water quality was not monitored more research should be done to be certain that this is true, and if it is, why that may be.

Acknowledgements

Thanks to Dr. Alicia Ebert and the Ebert Lab, Dr. Christine Vatovec, and Dr. Jana Kraft. Thanks to the University of Vermont's Undergraduates Research Opportunities Program for providing funding. Thanks to Alan Howard for statistical support.

Works Cited

Berninger, J. P., Du, B., Connors, K. A., Eytcheson, S. A., Kolkmeier, M. A., Prosser, K. N., Valenti, T. W., Chambliss, C. K., Brooks, B. W. (2011). Effects of the antihistamine diphenhydramine on selected aquatic organisms. *Environmental Toxicology, 30*(9), 2065-2072. doi:10.1002/etc.590

Boutaud, O., Aronoff, D.M., Richardson, J. H., Marnett, L. J., Oates, J. A. (2002). Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H2 synthases. *Proceedings of the National Academy of Science of the United States of America, 99*(10), 7310-7135. doi:10.1073/pnas.102588199

Brandhof, Evert-Jan van den, & Montforts, M. (2010). Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. *Ecotoxicology and Environmental Safety, 73*, 1862-1866. doi:10.1016/j.ecoenv.2010.08.031

Brooks, B. W., Turner, P. K., Stanley, J. K., Weston, J. J., Glidewell, E. A., Foran, C. M., Slattery, M., La Point, T. W., Huggett, D. B. (2003). Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere, 52*(1), 135-142. doi:10.1016/S0045-6535(03)00103-6

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters, 142*(3), 185-194. doi:10.1016/S0378-4274(03)00068-7

Dal Pan, G. J. (2009). *Acetaminophen: Background and Overview* (PDF presentation). Retrieved from https://www.fda.gov/downloads/Advisor.../UCM175767.pdf

Daughton, C. G. (2003). Cradle-to-Cradle Stewardship of Drugs for Minimizing Their Environmental Disposition While Promoting Human Health. I. Rationale for and Avenues toward a Green Pharmacy. *Environmental Health Perspectives, 111*(5), 757-774.

David, A., Pancharatna, K. (2009). Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish *Danio rerio*. *Journal of Applied Toxicology*, *29(7),* 597-602. doi: 0.1002/jat.1446

Ferguson, P. J., Bernot, M. J., Doll, J. C., & Lauer, T. E. (2013). Detection of pharmaceuticals and personal care products (PPCPs) in near-shore habitats of southern Lake Michigan. *Science of The Total Environment, 458-460*, 187-196. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.04.024

Grabicova, K., Lindberg, R. H., Ostman, M., Grabic, R., Randak, T., Larsson, D. G. J., & Fick, J. (2014). Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. *Science of The Total Environment, 488-489*, 46-50. doi:10.1016/j.scitotenv.2014.04.052

Hill, A. J., Teroaka, H., Heideman, W., Peterson, Richard E. (2005). Zebrafish as a Model Vertebra for Investigating Chemical Toxicity. *Toxicological Sciences, 86*(1), 6-19. doi: 10.1093/toxsci/kfi110

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M. T., Tanguay, R. L., Dong, Q. (2010). Toxicity, uptake kinetics and behavior assessment in zebrafish

embryos following exposure to perfluorooctanesulphonic acid. *Aquatic Toxicology, 98*(2), 139-147. doi:http://dx.doi.org.ezproxy.uvm.edu/10.1016/j.aquatox.2010.02.003

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics, 203*(3), 253-310. doi:10.1002/aja.1002030302

Kinney, C. A., Furlong, E. T., Werner, S. L., & Cahill, J. D. Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environmental Toxicology and Chemistry, 25*(2). doi:10.1897/05-187R.1

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance *Environmental Science & Technology, 36*, 1202-1211.

Novartis Pharmaceuticals Corporation, (2009). Tegretol-carbamazepine USP. Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/016608s101,018281s048lbl.pdf

Oetken, M., Nentwig, G., Loffler, D., Ternes, T., & Oehlmann, J. (2005). Effects of Pharmaceuticals on Aquatic Invertebrates. Part I. The Antiepileptic Drug Carbamazepine. *Archives of Environmental Contamination and Toxicology, 49* (3), 353-361. doi:10.1007/s00244-004-0211-0

Sanders, G. E. (2009). *Zebrafish Workshop: Water Quality Parameters and Fish Disease* (PDF Presentation). Retrieved from https://www.was.org/documents/MeetingPresentations/AA2009/AA2009_0822.pdf

Selderslaghs, I. W. T., Blust, R., Witters, H. E. (2012). Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryo toxicity using a training set of 27 compounds. *Reproductive Toxicology, 33(2),* 142-154. doi: http://dx.doi.org/10.1016/j.reprotox.2011.08.003

U.S. Food and Drug Administration, (2013). Diphenhydramine Hydrochloride Injection, USP. 2. Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/091526lbl.pdf

Vatovec, C., Phillips, P., Van Wagoner, E., Scott, T., & Furlong, E. (2016). Investigating dynamic sources of pharmaceuticals: Demographic and seasonal use are more important then down-the-drain disposal in wastewater effluent in a University City setting *Science of The Total Environment* doi:http://dx.doi.org/10.1016/j.scitotenv.2016.07.199

Wang, C., Shi, H., Adams, C. D., Gamagedara, S., Stayton, I., Timmons, T., & Ma, Y. (2011). Investigation of pharmaceuticals in Missouri natural and drinking water using high performance liquid chromatography-tandem mass spectrometry *Water Research, 45*(4). doi:http://dx.doi.org/10.1016/j.watres.2010.11.043