THE EFFECTS OF CARBAMAZEPINE ON THE DEVELOPING *XENOPUS LAEVIS* LIVER

A Report of a Senior Study

by

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Major: Your Major

Biology

Maryville College

Spring, 2025

Date approved _____, by _____

Faculty Supervisor

Date approved _____, by _____

Division Chair

ABSTRACT

Current municipal water treatment protocols do not call for specific filtration of pharmaceuticals from the treated water. As a result, many pharmaceuticals including carbamazepine (CBZ - a drug commonly indicated for epilepsy, bipolar disorder, and pain), are present in municipal and surface water systems. Prolonged exposure to CBZ has been shown to induce liver injury in study animals. Kupffer cells (KCs) are the liver's first line of immune defense, and hepatocytes (and cholangiocytes) are involved in the regeneration of the liver following physical or chemical liver injury. Thirty *Xenopus laevis* tadpoles were used to study the effects of prolonged exposure to CBZ on the livers of the 15 experimentals (which were exposed for 44 days to a dose of 5.3 x 10-4 L CBZ/1 L of water, the concentration of CBZ in tap water, and histological slides were made to study the livers of the tadpoles. Results showed a significant reduction in the average number of the experimentals' KCs vs. those of the controls ($p<0.0001$). Likewise, the average diameter of the experimentals' hepatocytes was reduced ($p=0.01688$). CBZ did not have a significant effect on the weight ($p=0.59719$) and number of deaths ($p=0.59369$). These results support the need for increased ongoing efforts to treat municipal water for pharmaceuticals.

ACKNOWLEDGEMENTS

I would like to thank Dr. Sharon May and the Senior Study grant committee for the funds which financed this study. Dr. Drew Crain: thank you for your tutelage and great patience during the process of this study. To my husband, Scott Susong and my son Josiah Susong, thank you both for your support and encouragement during my studies. Dr. Angelia Gibson – I could not have had a better advisor. And above all, I thank my Lord and Saviour, Jesus Christ for bringing me thus far, and for the many great things that are ahead in my life, studies, and vocation.

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CHAPTER I

INTRODUCTION

The medical renaissance included a great number of accomplished physicians and surgeons who made especial contributions to our knowledge of human anatomy and physiology; Vesalius assembled detailed anatomical information; Paré advanced surgical techniques; and Harvey, a medical genius, detailed the circulatory anatomy and physiology Toledo-Pereyra (2015). Unfortunately, as with many human activities, these advances have come at a cost, namely in unwanted side effects in some people, and just as importantly, on the environment – specifically water and the wildlife that lives in which can become contaminated with pharmaceuticals (see Table 1). After absorption and action, all drugs are eliminated as waste. After excretion or being discarded, pharmaceuticals enter waterways after passing through municipal waste treatment facilities (Carlton 2023). Wastewater treatment plants can trap and remove some pharmaceuticals, but smaller ones often pass through the filtration/treatment process, and that's how they end up in rivers and streams (Clark et al., 2004).

One such pharmaceutical is Carbamazepine (CBZ), a pharmaceutical used for the treatment of epilepsy, bipolar disorder, and neuropathic pain. Because of its small size (molecular weight of 236.27 g/mol), CBZ is easily absorbed by aquatic organisms such as amphibians, especially at the larval stage. It has been shown to have harmful physiological and behavioural effects on amphibians at various stages of development. Because amphibians are prey to many land and water predators, whatever they ingest or absorb into their systems becomes part of the food web and is sometimes passed onto their predators (Guo et al., 2023).

Water Treatment

Undigested material that enters the gastrointestinal tract and some waste products of metabolism are eliminated in the feces (Guyton and Hall, 2006). The waste either goes to a public sewage treatment plant or the septic tank for those homes which don't have public sewer service. Once at the water treatment plant, the sewage/raw water undergoes the following initial processes which progressively filters out particulates from the biggest to the smallest:

- Source water collection
- Coagulation and flocculation
- Sedimentation
- Filtration
- Disinfection

A multi-stage system comprising gravel pre-filters, slow sand filters and terminal disinfection conforms with the multiple barrier concept in relation to pathogen removal (Clark et al., 2004). Additional steps including oxidation, photolysis, UV-degradation, nanofiltration, reverse osmosis, and adsorption are used in an attempt to rid the water of remaining contaminants such as chemicals (Patel et al., 2019). The steps above are generally effective at removing many pharmaceuticals/chemicals, particulate matter and disease-causing microbes (Clark et al., 2004); however, they fail to remove many pharmaceuticals, which are too small to be trapped by the multi-level filtration system and are unaffected by the disinfection process. Psychoactive pharmaceuticals like carbamazepine comprise the highest concentration of pharmaceuticals in domestic wastewater (Subedi et al., 2013). This combined with the fact that they are too small to filter out or adsorb through the water treatment protocols, means that carbamazepine ends up in the world's ground and surface waters, and is even detectable in sea water (Patel et al., 2019).

Wastewater Pharmaceuticals and the Freshwater and Marine Food Web

The concentration levels of pharmaceuticals in drinking water are presently deemed acceptable per current water treatment standards, given our size and physiology, however they are problematic on both freshwater and marine life due to the small size of most of the organisms that live in the water (Carlton 2023). This is also due to the thin skin of most water-dwelling organisms and the fact that most easily absorb substances (including pharmaceuticals) through their membranous skin (Brown 2010). These organisms have

extensive prey-predator relationships, and the pharmaceuticals absorbed or ingested by the prey end up in the systems of the predators, and on up the food chain. Figure 1 shows an example of this using diatoms, which are part of the diet of water dwellers such as *Xenopus laevis*. Figure 2 illustrates how pharmaceutical-laden water reaches flora and fauna, contaminating the food web.

Table 1. Top Pharmaceuticals in Drinking Water

Information from Claude AI (2024)

Pharmaceutical	Common Use	Concentration (µg/L)	Citation
Metformin	Anti-diabetic	1.0 - 10.0	Scheurer, M. et al. (2012)
Caffeine	Stimulant	0.1 – 5.0	Buerge, I.J. et al. (2003
Carbamazepine	Anti-convulsant	0.1 - 2.0	Zhang, Y. et al. (2008)
Ibuprofen	NSAID	0.01 - 1.0	Fent, K. et al. (2006)
Diclofenac	NSAID	0.01 - 0.5	Heberer, T. (2002)
Naproxen	NSAID	0.01 - 0.5	Buser, H.R. et al. (1999)
Gemfibrozil	Lipid regulator	0.01 - 0.5	Ternes, T.A. et al. (2002)
Sulfamethoxazole	Antibiotic	0.02 - 0.4	Hirsch, R. et al. (1999)
Atenolol	Beta-blocker	0.01 - 0.2	Huggett, D.B. et al. (2003)
Fluoxetine	Anti-depressant	0.001 - 0.05	Brooks, B.W. et al. (2003)
Ethinylestradiol	Contraceptive	0.001 - 0.005	Kolpin, D.W. et al. (2002)

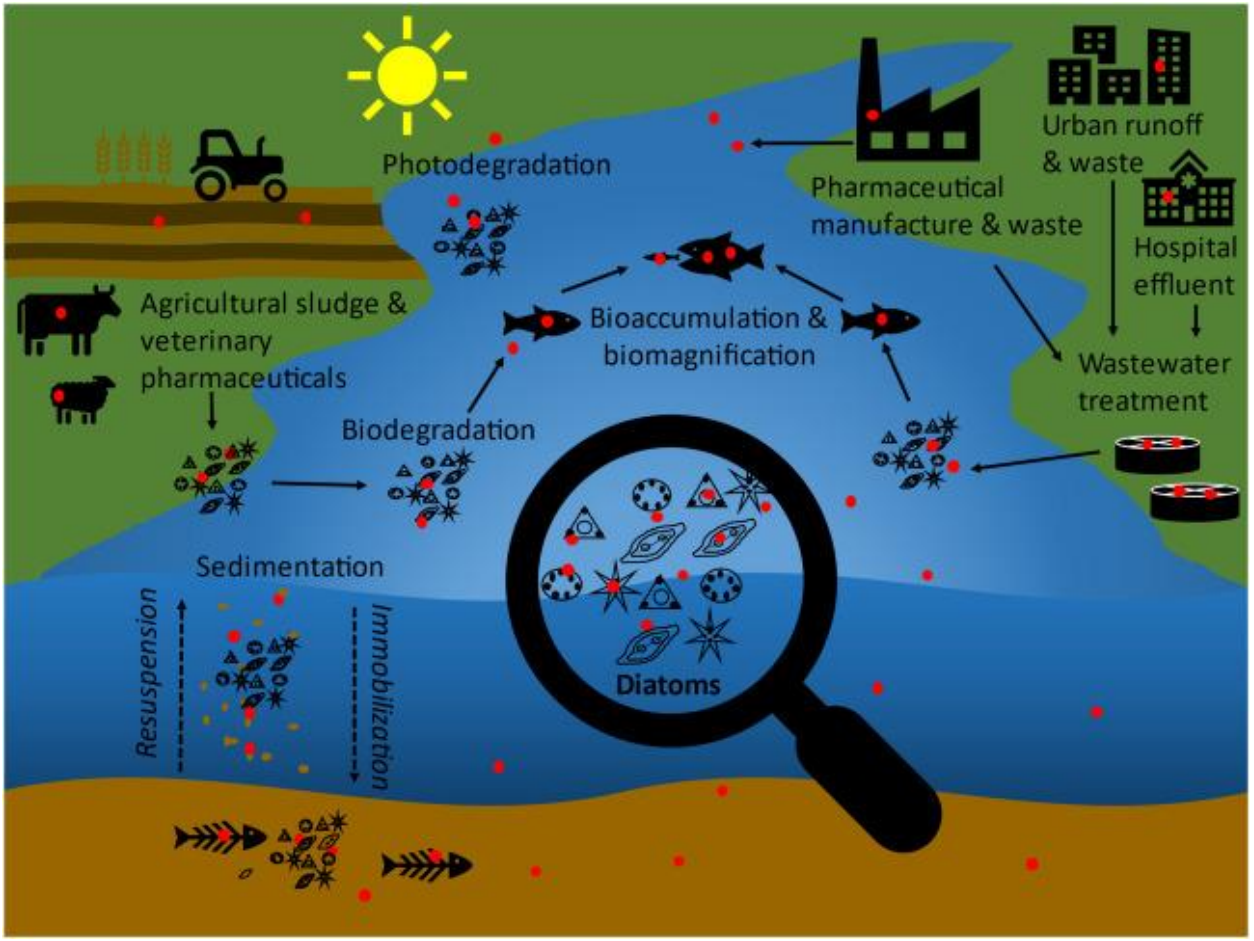

Fig. 1. The sources of rural and urban inputs of pharmaceuticals (solid red circles) into freshwater systems and the processes responsible for the breakdown (photodegradation, biodegradation), accumulation and mobility (sedimentation, immobilisation/adsorption, resuspension/desorption, bioaccumulation) and biological impacts (bioaccumulation, biomagnification) of pharmaceuticals in the aquatic environment. (Kock et al. 2023, pg. 3)

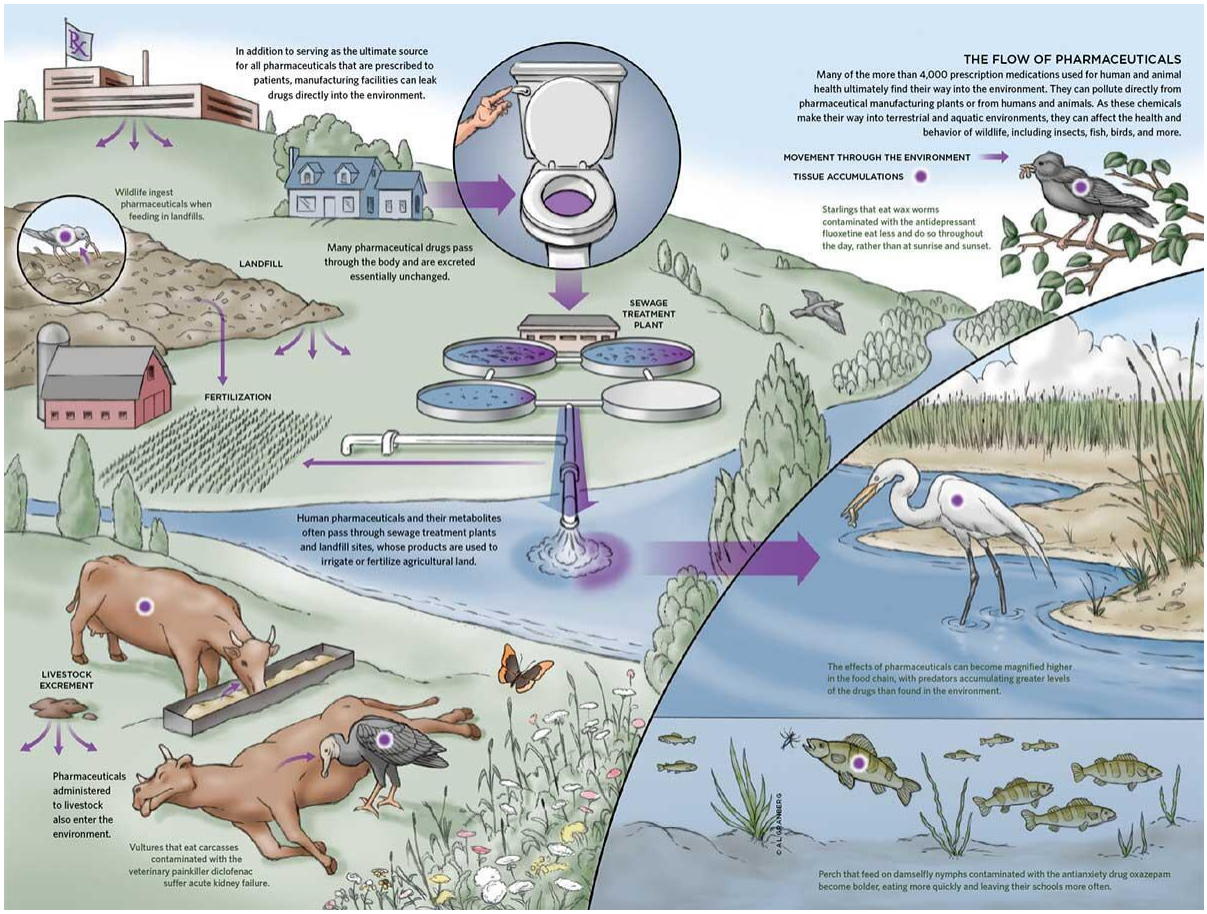

Fig 2. The Flow of Pharmaceuticals

Many of the more than 4,000 prescription medications used for human and animal health ultimately find their way into the environment. They can pollute directly from pharmaceutical manufacturing plants or from humans and animals. As these chemicals make their way into terrestrial and aquatic environments, they can affect the health and behavior of wildlife, including insects, fish, birds, and more (Granberg 2018).

Xenopus laevis as a Model Organism

To be a model organism, an animal must: have a well-studied cell structure and physiology, have a well-documented genome, be relatively easy to acquire and maintain in a laboratory, and relatively easy to administer pharmaceuticals to study their effects within a reasonable timeline (Hughes & Dyer 2018, and Muller & Grossniklaus, 2010). The African clawed frog (*Xenopus laevis*) has long been used as a model organism for various fields of study such as genetics, cell biology, physiology, and pharmacology. As with other amphibians, during fertilization, the male releases sperm to fertilize the eggs as the female is releasing them from her body (Sive & Harland 2023). The embryos hatch within a couple of days and their relatively large size makes them easier to handle and to study as they go through the various embryonic stages (Harland & Grainger, 2011).

Despite being an amphibian, *Xenopus* morphology and physiology are comparable to those of humans. This makes *Xenopus* ideal for modeling studies of various human organs such as the liver, brain, etc. It also makes it ideal for studying disease and the efficacy of various pharmaceuticals used to treat human diseases, and the side effects of those pharmaceuticals (Hellsten et al. 2010). Figure 3 shows the developmental stages of *Xenopus laevis* larvae.

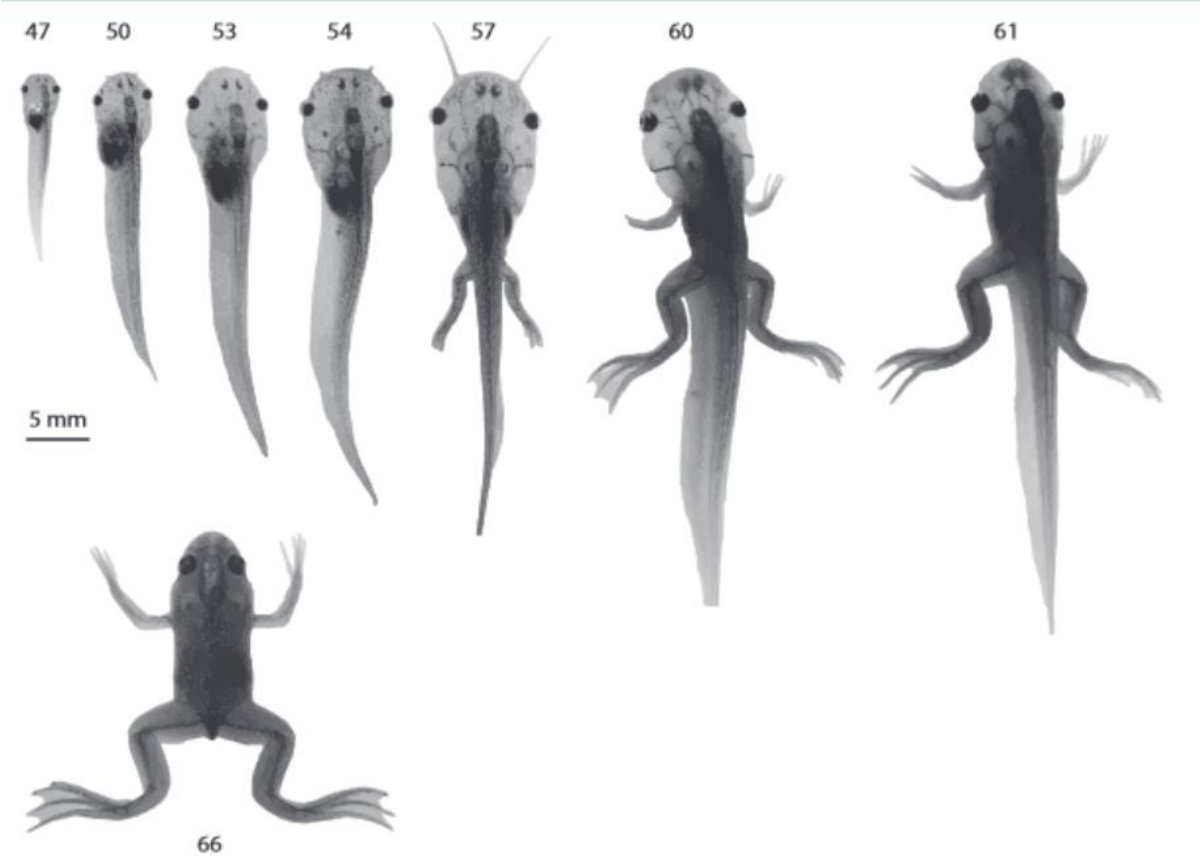

Fig 3. An overview of late-stage *Xenopus laevis* tadpoles (Hänzi, et al., 2016).

The Liver

With over 500 functions, the liver is the largest and busiest organ in the body (Ishibashi et al. 2009). Some of its functions include detoxification of xenobiotics (such as Carbamazepine), protein synthesis, hematopoiesis, homeostasis, immune response, etc. (Holzhütter et al. 2012). The liver is capable of regenerating itself. It is the only solid organ

that uses regenerative mechanisms to ensure that the liver-to-bodyweight ratio is always at 100% of what is required for body homeostasis (Michalopoulos 2021). Structurally and histologically, the liver can be divided into 5 tissue systems: 1) vascular system, 2) hepatocytes and hepatic lobule, 3) hepatic sinusoidal cells, 4) biliary system, and 5) stroma (Ishibashi et al. 2009). My research will focus on the Von Kupffer cells, which are involved in immunity, and the hepatocytes, which give rise to the liver itself.

Von Kuppfer Cells

Von Kupffer cells (named after their discoverer, Karl Wilhelm von Kuppfer) are macrophages specific to the liver and are the liver's primary immune defense system (Nguyen-Lefebvre and Horuzusko 2015). They are also known as Kuppfer cells (KCs). There are different types of KCs with different physiological and pathological functions, which makes them a target for various immune therapies for various liver ailments (Nguyen-Lefebvre and Horuzusko 2015). KCs can become leucocytic in function and produce leukotrienes when attacking cancer cells and diseased hepatocytes within the liver (Wardle 1987). Additionally, they scavenge LDL lipoproteins from the blood and produce lymphokine mediators that direct protein synthesis by the hepatocytes (Wardle 1987, Panin et al. 1994).

Hepatocytes

Hepatocytes are the main cells in the liver and perform most of the many functions of the liver mentioned above. Since the liver is the main detoxification organ of the body, hepatocytes play a big role in the removal of xenobiotics (Rogiers & Vercruysse 1993). Excessive and/or prolonged exposure to xenobiotics during fetal development can induce biotransformation of the hepatocytes depending on the embryonic stage and length of exposure (Kühnert et al. 2017). Hepatocytes, or liver cells, detoxify substances through a three-phase process:

Phase I: Toxic substances are modified through reactions like oxidation, reduction, hydrolysis, hydration, and dehalogenation. These reactions are primarily facilitated by cytochrome P450 enzymes.

Phase II: The intermediates created in phase I are further processed by binding them to endogenous molecules like glutathione, sulfate, or amino acids. This makes them more water-soluble and less harmful.

Phase III: The conjugated toxins are transported and eliminated out of hepatocytes, primarily into the bile or urine for excretion (Google Generative AI 2024).

Research Question

My research question is, how does Carbamazepine affect the development and function of the larval liver? My hypothesis is that there will be some structural differences between the livers of the control tadpoles and the ones of the experimentals.

CHAPTER II

METHODS & MATERIALS

Xenopus laevis tadpoles (n=30) stages 60 -63 were purchased from Xenopus express (xenopus.com). All husbandry and experimental protocols were approved by the Maryville College IACUC (see Appendix 1). Five tadpoles were placed in 6 separate 5.5-gallon fish tanks (3 control and three experimental). We prepared 2ml of 3mg/ml carbamazepine (CBZ) in 95% ethanol using the following method:

a. We weighed 6 mg (i.e. 0.006 g) and added 2 ml of ethanol
b. We vortexed
c. We filled 6 - 5.5-gallon fish tanks with 4 L each of dechlorinated water.
d. We added 530 µl of 95% ethanol to the three control tanks, and 530 µl of our CBZ stock to each of the other three tanks: our experiments. This resulted in an exposure dose of 5.3×10^{-4} L CBZ/1 L of water.

e. We added 5 Xenopus laevis tadpoles at various stages of development to each tank, then added a pinch of aquatic pet food and observed.

Tanks were changed and tadpoles fed every other day one tank at a time. We made note of their different developmental stages and behaviour (see Appendix 2). Most noticeable was that after a week, the experiments were more hyper when being handled. Some would have jumped out of the net if we didn't have a second net over it. As a precaution, started lining the sink drain with a wet paper towel and placing a net to create a barrier in case one successfully jumped out of the net. We rinsed the sink and net and used a new paper towel for each tank. We opened the abdominal cavities of any tadpoles that died and fixed them in Bouin's fixative solution.

We exposed the tadpoles for 44 days. On day 44, starting with the Ctrl 1 tank, we placed all frogs in a watch glass filled with MS-222 (tricaine methanesulfonate) to anesthetise them. The solution was 2g/L MS-222 + 1.5 g $NaHCO_3$(sodium bicarbonate). Each frog was then removed from the solution, placed on a weigh boat on a scale and weighed, then still on the weigh boat, was placed under a dissection microscope, and dissected at the abdominal cavity. The liver was removed, placed in a bullet tube, and fixed in Bouin's solution. Controls were labeled as follows: C1= Crtl 1, 1 = Frog 1, L = liver; E1, etc. was used for the Exps.

The above steps were repeated for each frog, and the bullet tubes were labeled accordingly. The next day, the Bouin's was poured out into an old jug under the hood vent

and the jug was sealed. We used 70% ethanol to clear tissues of Bouin's solution. The ethanol & old Bouin's solution was poured into an old jug, which was then sealed. Samples were left in the ethanol for 4 weeks (over Christmas break).

Embedding

After clearing, tissues were embedded. The samples were then taken out of the tube, placed into a tissue cassette and labeled. The cassette was then placed into an 80% ethanol jar for 1 hour, then into a 95% ethanol jar for 1 hour, then a 100% ethanol jar for 1 hour, then a 100% ethanol jar for 1 hour, then a "Safeclear 1" jar for 1 hour and finally the "Safeclear 2" jar for 1 hour. The cassette was then placed into the "Wax 1" jar in the vacuum oven. The pressure was changed to 12 Hg and the cassette was left in the jar for 1 hour. Using heated forceps, the cassette was then placed in the "Wax 2" jar for 1 hour with a pressure of 15 Hg. Using heated forceps, the cassette was then placed in the "Wax 3" jar for 1 hour with a pressure of 21 Hg. Using heated forceps, the cassette was then placed in the "Wax 4" jar for 1 hour with a pressure of 25 Hg. Obtained a new cassette and a cassette holder. Placed a drop of glycerol into the holder. Poured wax into the holder and placed the samples in the wax. The new cassette was placed on top of the wax and the wax was topped off. They were then left overnight to dry.

The samples were then sectioned into 12 µm (1 cell) thick slide length ribbons using a ThermoScientific Shandon Finesse 325 microtome. The slices were then placed into a

warm water bath with a pinch of gelatin added. Using a spatula, the ribbons were placed on individual slides and labeled. The slides were left to dry on a slide warmer overnight.

The slides were stained under the hood with Hematoxylin and Eosin according to **Figure 1**. After staining, permount adhesive was used to attach a coverslip to each slide. The slides were left to dry overnight. They were then placed in a slide box and the box was kept vertical to prevent the tissues from sliding off the slide.

The slides were examined under a microscope in order to compare the experiments versus the controls and to determine the best quality slides to use for analysis.

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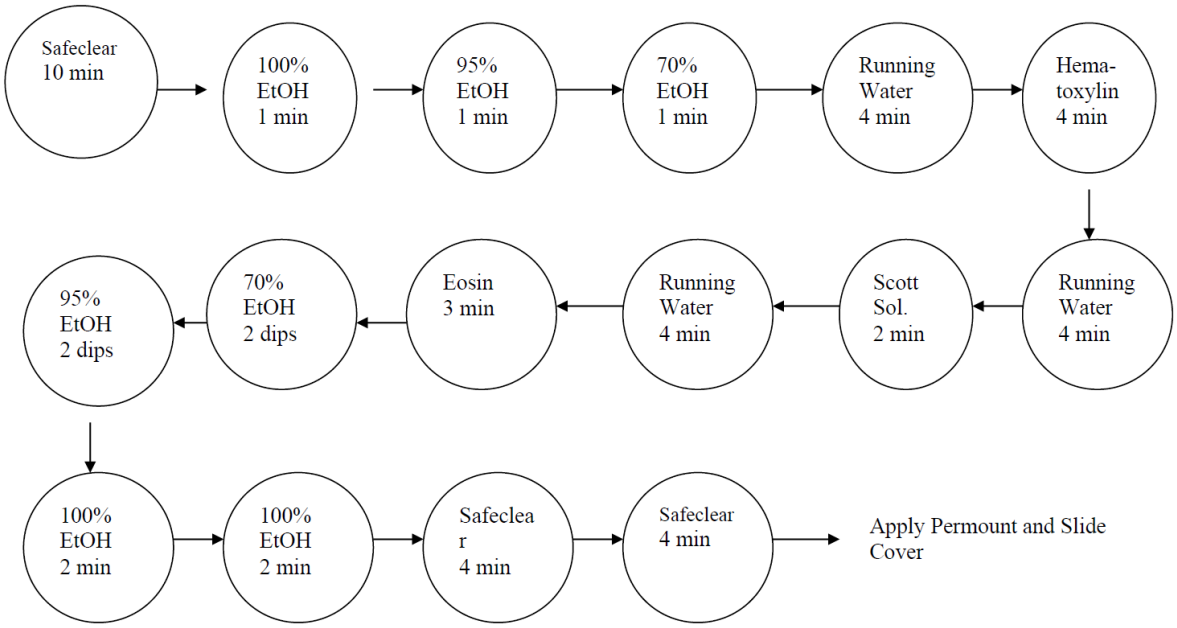

Figure 4. This figure shows the steps taken to stain the slides containing the ribbons of the liver tissue.

Kupffer cells were counted per microscope field of view under 100x magnification. Because of challenges producing an equal number of viable slides per frog, I counted the number of quality views for controls vs. quality views per experiments as a whole. Only good quality samples from each tank that could fill the field of view on the monitor were used for both the controls and the experiments. An Excel spreadsheet was created to log the counts for analysis.

The diameters of hepatocytes (40 per slides from tadpoles in each of the tanks, so 120 from controls and 120 from experiments) were measured under the microscope at 400x magnification and recorded in an Excel spreadsheet for two-tailed t-test analysis. As with the counting of the Kupffer cells above, I took the measurements on a quality-of-slides basis, using only the best quality slides from each tank. Also, pictures of hepatocytes and Kupffer cells were taken under the microscope at 400x and 100x magnifications, respectively.

Statistical Analyses

Two-tailed t-tests were performed for both the hepatocytes and Kupffer cells, and the results were used to calculate the p-values.

CHAPTER III

RESULTS

Macroscopic Results

There was no difference in average weight at anaesthetisation between the controls (1.12 g) and the experimentals (1.07 g) ($p=0.59719$). This is illustrated in Figure 5.

Six deaths occurred within eight days of the start of the experiment, with all three of the control deaths occurring by day 6. Three of the experimentals deaths occurred on the same day or eight days after initial exposure to CBZ. There were no deaths from either the controls or experimentals after day 8.

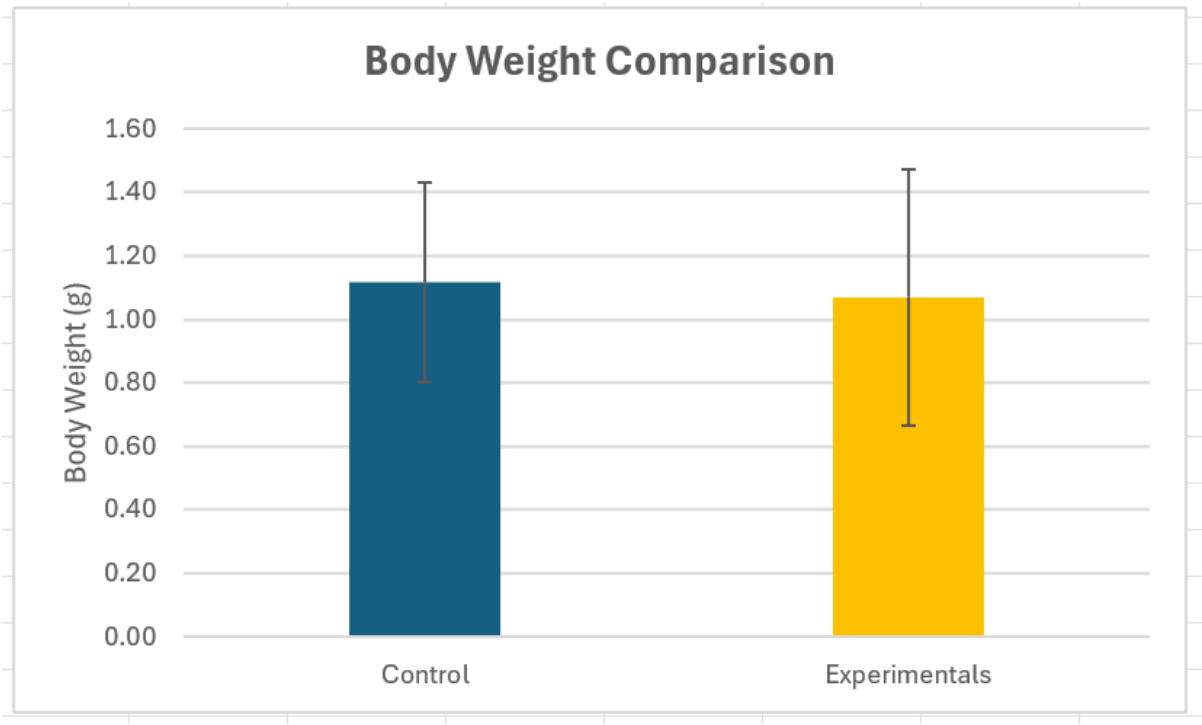

Figure 5 Mean difference (+/- SE) in body weight at anaesthetisation.

Microscopic Results

Both KCs and hepatocytes were significantly affected by carbamazepine (see figures 6-9). The averages for the number of KCs per field of view were 60.03 for the controls and 33.02 for the experimentals ($p<0.0001$). Similarly, the average diameter for the hepatocytes was 6.79 μm for the controls and 6.18 μm for the experimentals, and this was also significant ($p<0.0004$).

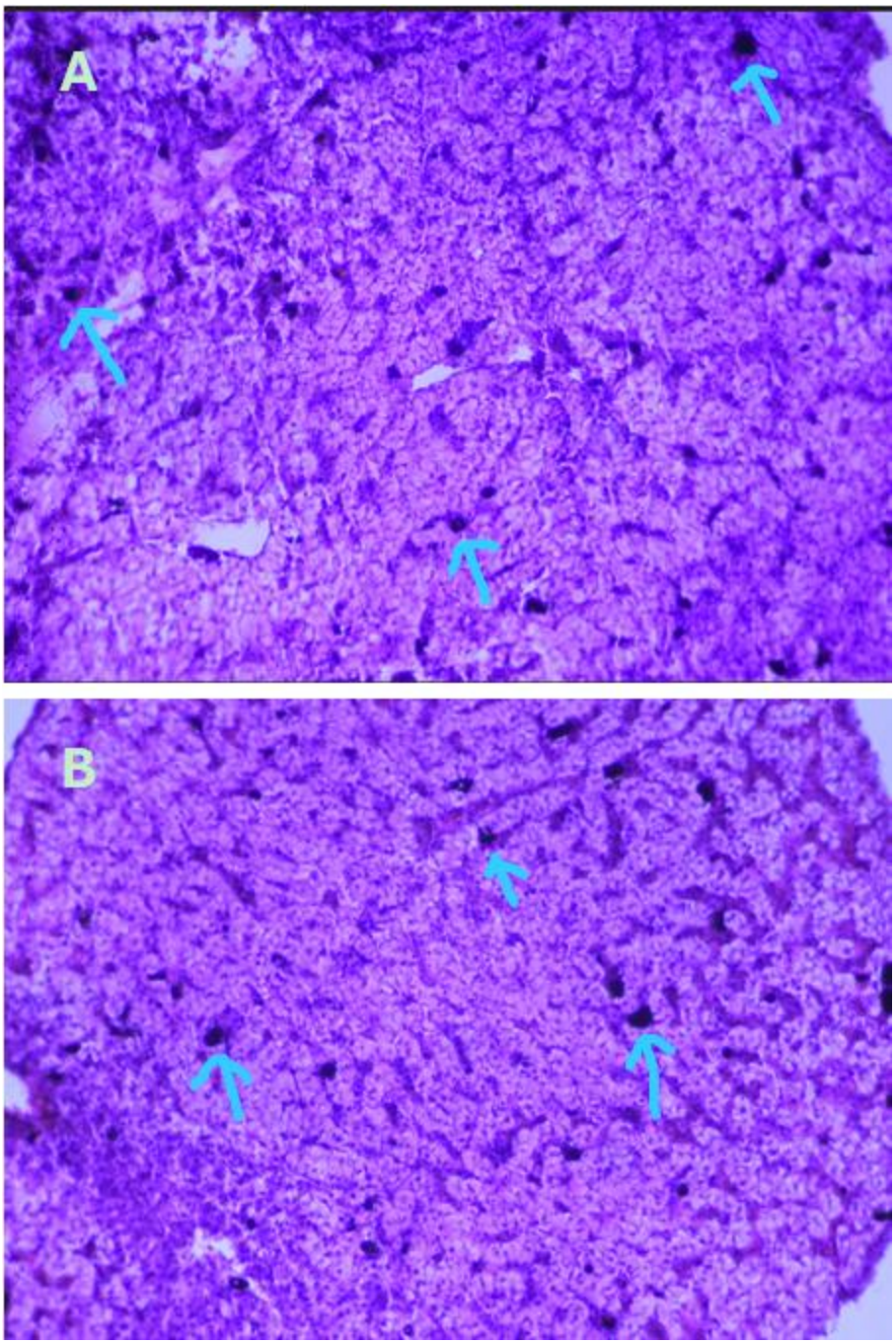

Figure 6: Kupffer Cells (A: Control and B: Experiment) at 100x Magnification.

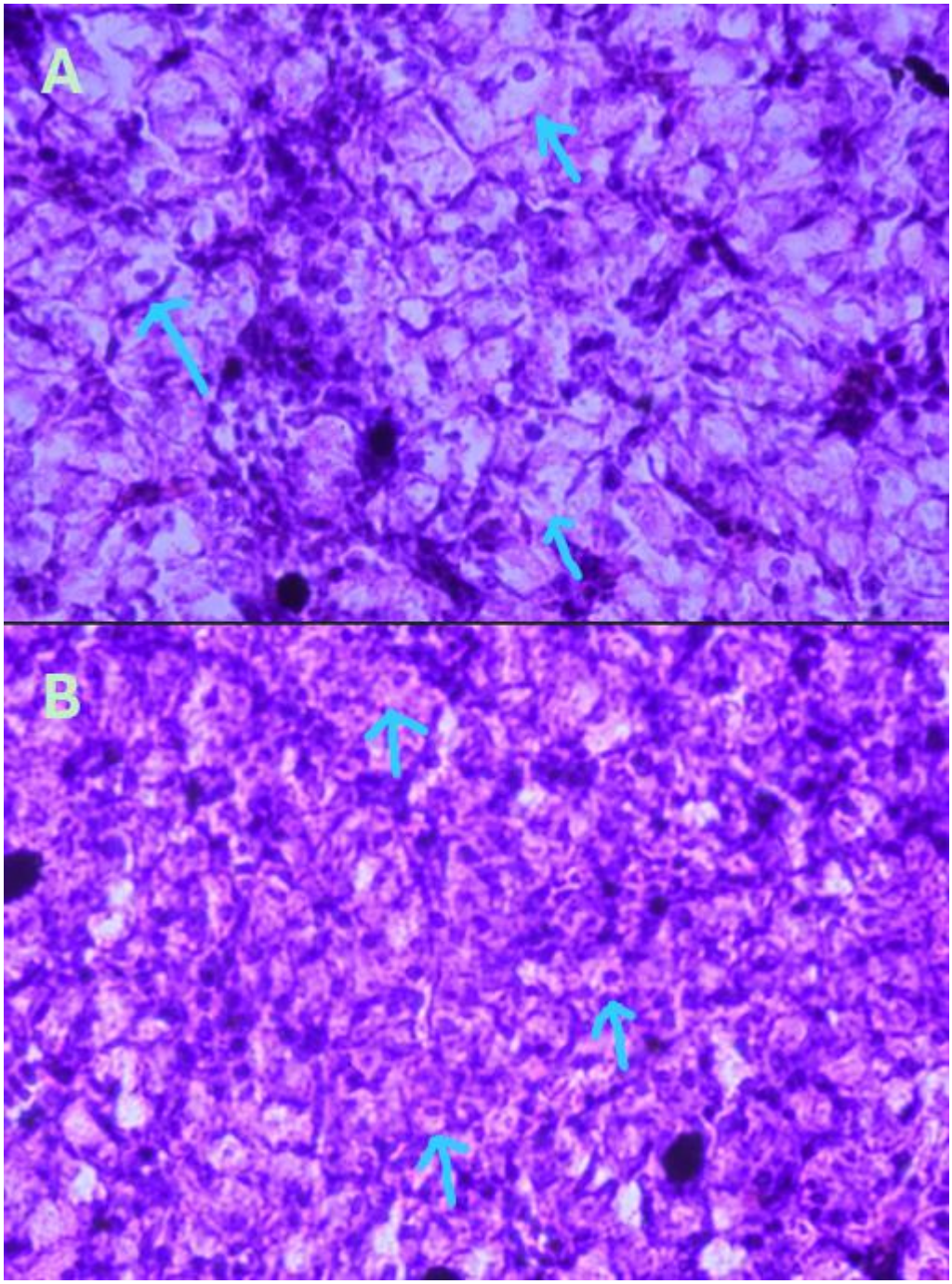

Figure 7: Hepatocytes (A: Control and B: Experiment) at 400x Magnification.

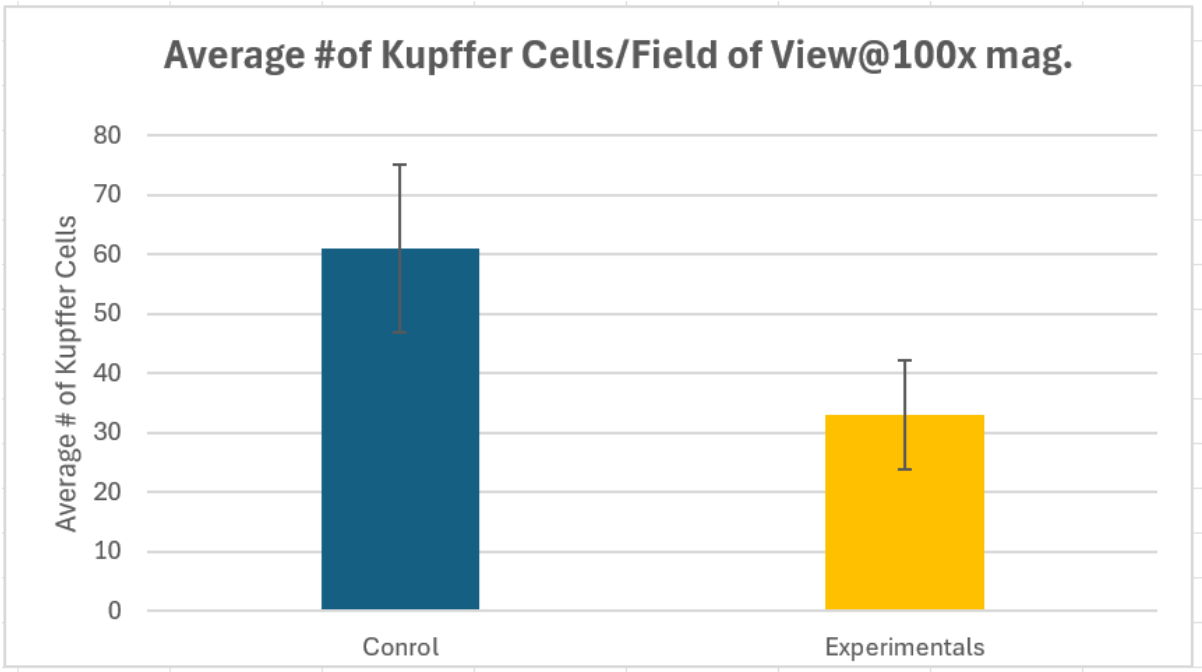

Figure 8: Average # of Kupffer cells in a Field of View at 100x Magnification for Control and Experiment Liver Samples (+/- SE).

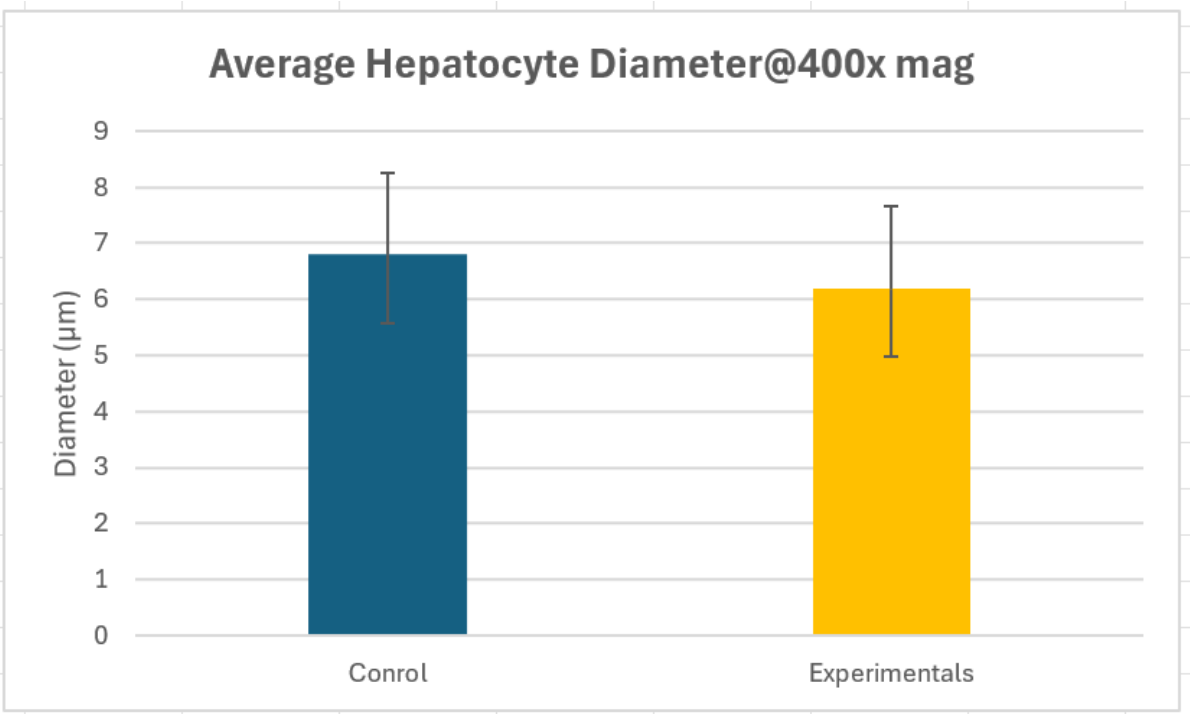

Figure 9: Average Diameter of Hepatocytes in a Field of View at 400x Magnification for Control and Experiment Liver Samples (+/- SE).

Qualitative Results

Overall, the more developed the tadpoles got, the more active they were, but the experimentals were in general more hyperactive than the controls. The behavioural differences were most noticeable during water changes within two days of CBZ exposure, with a couple of the experimentals almost jumping out of the net. The experimentals also seemed to spend more time at the water surface than did the controls. The limbs of the experimentals appeared overall less developed and shorter than those of the controls. One experimental frog showed especially underdeveloped limbs compared to the other experimentals. The intestine was also smaller, and had a darker colour (dark pink and grey).

CHAPTER IV

DISCUSSION

The hypothesis that carbamazepine (CBZ) would have an effect on the number of KCs and on the diameter of the hepatocytes was supported as the average number of KCs in the controls was significantly greater than the number in the experiments, likewise for the average hepatocyte diameter of the controls vs. the experiments. Because KCs are in the first line of defense of the liver's immune and detoxification system, they naturally present with more pronounced negative clinical manifestations in the process of performing their function. Other experiments have shown that embryo-derived KCs (EmKCs) accumulate large amounts of lipoprotein-derived cholesterol, in part through the scavenger receptor CD36, and massively expand early after the induction of hypercholesterolemia (Fima et al. 2024). The Kupffer cells-mediated inflammation was predominant in the development of the CBZ-induced liver injury in rats. (Sasaki et al. 2026). Oxidative stress caused by early prolonged exposure and extended immune response to CBZ may be the cause of the drastic reduction in the number of KCs observed in the experiments. After this rapid adaptive response, EmKCs exhibit mitochondrial oxidative stress, and their numbers gradually diminish while monocyte-derived KCs (MoKCs) with reduced cholesterol-loading capacities seed the KC pool (Fima et al. 2024).

Hepatocytes (and cholangiocytes) are responsible for the regeneration of the liver after damage, and when regeneration of one of the two cell types fails, hepatocytes and cholangiocytes function as facultative stem cells and transdifferentiate into each other to restore normal liver structure (Michalopoulos, 2021). It is possible that this process is one of the reasons for the smaller average size of the hepatocytes – perhaps the smaller ones are differentiated from cholangiocytes instead of from hepatocytic stem cells. Visually, the histology of the experimentals shows their hepatocytes not only being generally smaller than those of the controls, but also being densely packed together. This might be explained by the hepatocytes' reparative role in the liver. However, upon physical or chemical injury to the liver, hepatocytes proliferate extensively in vivo under the direction of multiple extracellular cues, including Wnt and pro-inflammatory signals (Peng et al. 2018). Both genetic and epigenetic effects of developmental exposure to xenobiotics in the aquatic environment has been shown to reduce both the liver size and number of hepatocytes in a dose-dependent manner (Xiong et al. 2023). One study's results demonstrated that BPA exposure disturbed lipid metabolism, and induced oxidative stress, ERS, apoptosis, autophagy and inflammatory response in the liver of common carp (Gu et al. 2021). Both findings can be inferred to the number of KCs and size of hepatocytes. There are studies that have shown that hepatocyte size varies depending on the region/zone of the liver that they are found. … results indicate that differences in cell size among isolated human hepatocytes are related to zonal origin, and that elutriation-based size fractionation enables studies of zonated human liver functions in

vitro (Ölander et al. 2021). Zonation was not taken into account in measuring hepatocyte diameters for this study, and is advisable for future studies.

CBZ did not show a significant effect on the tadpoles' weights and number of deaths. Perhaps a longer study might yield different results. No postmortem of the tadpoles that died during the experiment was performed to compare their livers to the ones of those anesthetised after the study period: in hindsight, this could have yielded some useful results. Also using a different model study animal that is easier to weigh throughout the study would be advisable for future studies.

Other considerations to be made for future studies include a much larger sample size, perhaps parallel experiments using different model organisms to see if the effects observed in this study are similar across the board. The involvement of metabolic activation was demonstrated in developing CBZ-induced liver injury, and a difference in metabolic activation reactions between mice and rats was indicated (Sasaki et al. 2016). Testing all subjects prior to the study onset to rule out genetic abnormalities and/or preexisting liver conditions is also advisable for future studies.

The implications of this study's results run the gamut from bioaccumulation of CBZ and other pharmaceuticals in tandem with other contaminants like microplastics present in our water sources, to the way pharmaceuticals are manufactured, and to the public health policies currently in place, which deem the present levels of chemicals in our water acceptable. It is hoped that pharmaceutical companies can voluntarily and collectively work together to figure out a way to attach large inactive ingredients onto pharmaceuticals with

small molecular weights to help increase the size of the pharmaceuticals' molecules so they can be easily filtered out by the water treatment plants' filtration systems. The data from this study support other studies suggesting that prolonged exposure to xenobiotics and pharmaceuticals such as CBZ, which are not effectively filtered out of municipal water systems, causes chemical injury to biological cells and systems.

APPENDICES

APPENDIX 1: Signed IACUC approval form for use of live animals in this study

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

Provide information after each bold item
Student Name:
Madison Breazeale and Lisa Nkala
Student Email Address:
madison.breazeale@my.maryvillecollege.edu, lisa.nkala@my.maryvillecollege.edu
Date:
August 29, 2024
Senior Study Advisor:
Dr. Crain
Species to be used:
Xenopus laevis
Age of animals:
Stage 50 through metamorphosis

Number of animals in study:
50
Duration of study:
October, 20024 - April, 2025
Location of animals during the study (building and room):
Sutton 114

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Madison Breazeale	875-738-5010	same	same
Lisa Nkala	678-923-4937	same	same

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.
Metamorphs will be euthanized with MS222 (2g/L, buffered with 1.5 g of NaHCO3).

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

Maryville College IACUC Approval Number: 202402
Date Approved: 08/29/24
Signed: Dr. David Clayton

APPENDIX 2: Daily Log

10/16/2024

We observed for change in behaviour after treating the water and adding food, there was none, except for when we initially inserted the pipette into the tanks to administer the treatments and lightly stirred the water to mix it in.

10/17/2024

Fed the tadpoles and observed. Controls 1 & 2 were calm. The tadpoles in Control (Crtl) 3 were more active. Experiment (Exp) 1 tadpoles seemed to be the most active, and they ate the most. Exp 2 were calm, and Exp 3 had a couple of more active ones. The more developed tadpoles (stagewise) seemed to be more active and ate more than the less developed ones. The tails of the less developed tadpoles exhibited twitching/vibrating movements.

10/18/2024

Removed waste, old food and 1 L of water from each tank and replaced with 1 L of fresh dechlorinated water. Had a one dead tadpole in Crtl 1 – fixed it in Bouin's solution.

10/19/2024

Checked on tadpoles – all doing well.

10/20/2024

Fed tadpoles. Lost one from Exp 1 -fixed it in Bouin's.

10/21/2024

The water was too cloudy to just vacuum the bottom of the tanks, remove and replace 200 ml of water with fresh dechlorinated water, so for each tank we transferred the

tadpoles to large beaker with some of the water that they were already in. We emptied and scrubbed the tank clean, then filled it with 4 L of fresh dechlorinated water. We added 530 µL of 95% ethanol to the Crtl tanks and 530 µL of the 95% ethanol and 0.06 g CBZ solution to the Exp tanks prior to returning the tadpoles to their respective tanks. Then. We observed the tadpoles and did not notice any behaviour that was out of the ordinary. The older tadpoles seemed more active than the younger ones. We counted the number of tadpoles in each tank: Crtl 1: 6, Crtl 2: 6, Crtl 3: 5, Exp 1: 4, Exp 2: 6, Exp 3: 6.

10/22/2024

One dead in Crtl 3. Removed from tank, cut open the abdominal cavity (neck to tail), and fixed in Bouin's solution under the hood vent). Labeled vial with the date and the tank that the tadpole came from.

10/23/2024

Changed and treated the water. Decided to include developmental stage (St) with our counts. Crtl 1: 5 grown/St 66, Crtl 2: 1 St 59, 1 St 63, 3 St 64. Crtl 3: 1 St 59, 1 St 63, 1 St 64, 1 St 65. Exp 1: 1 St 60, 3 St 63, 1 St 64 – more active than the controls (two almost jumped out of the net while being transferred). Exp 2: 1 St 61, 2 St 63, 2 St 65 – as active as Exp 1. Exp 3: 1 St 60, 3 St 64, 1 St 65. For each tank, we covered the tadpoles with a second net while transferring, and covered the sink drain with a wet paper towel in case any of the tadpoles jumped out of the net and landed in the sink. We rinsed the net and sink every transfer per tank.

10/24/2024

Checked on the tadpoles: 3 dead: 2 St 63 from Exp 1 and 1 St 62 from Exp 3. Removed from tank, cut open the abdominal cavity (neck to tail), and fixed in Bouin's solution under the hood vent). Labeled each vial with the date and the tank that each tadpole came from.

10/25/2024

All tadpoles were alive. Changed the water. All tadpoles hyper, especially the experiments. They all tried to escape during transfer and took a while to catch them with the net. Count and stages were: Crtl 1: 2 St 64, 1 St 65, 2 St 66. Crtl 2: 1 St 62, 3 St 65, 1 St 64. Crtl 3: 1 St 62, 2 St 65, 1 St 66. Exp 1: 3 St 65, 1 St 64, 1 St 65, 1 St 66. Exp 3: 5 St 65.

10/28/2024

Changed and treated water per protocol. Fed the tadpoles. The Exps were more hyper than the controls, especially the more mature ones. Count was as follows: Crtl 1: 1 St 65, 4 St 66. Crtl 2: 1 St 64, 1 St 65, 3 St 66. Crtl 3: 1 St 63, 1 St 65, 2 St 66. Exp 1: 1^{St} 64, 4 St 66. Exp 2: 1 St 65, 2 St 66. Exp 3: 5 St 66.

10/29/2024

One St 63 dead from Crtl 3. Fixed in Bouin's.

10/30/2024

Changed and treated water. Fed tadpoles. Count was: Crtl 1: 5 St 66. Crtl 2: 1 St 65, 4 St 66. Crtl 3: 1 St 64, 2 St 66. Exp 1: 1 St 65, 4 St 66. Exp 2: 3 St 66 – very active. Exp 3: 5 St 66 (2 possibly mating) – very active. The Exps swim to the surface more, and interact with each other more than the Crtls.

11/1/2024

Checked on the tadpoles – all alive and well. Growing.

11/2/2024

Changed water and fed tadpoles. All alive and well. Count: Crtl 1: 5 St 66. Crtl 2: 5 St 65 & St 66. Crtl 3: 3 St 66. Exp 1: 5 St 65 & St 66. Exp 2: 3 St 66. Exp 3: 5 St 65 & St 66. All seem hyper during transfer. St 65s swim very fast and avoid the net. St 66s try to jump out of the net.

11/3/2024

All tadpoles alive and well.

11/4/2024

Changed and treated the water. Fed tadpoles. Count: Crtl 1: 5 St 66. Crtl 2: 5 St 66. Crtl 3: 3 St 66. Exp 1: 5 St 66. Exp 2: 3 St 66. Exp 3: 5 St 66. All active. Exps swim to the surface more.

11/5/2024

All alive and well.

11/6/2024

Changed and treated water. Fed tadpoles. All alive and well.

11/7/2024

All alive and well.

11/8/2024

Changed and treated water. Fed. All hyper, but Exp 3s were the most hyper and tried to escape more. One escaped and was recaptured and placed back in the tank.

11/9/2024

All alive and well.

11/10/2024

All alive and well. Changed and treated water. Fed.

11/12/2024

Changed and treated water. Fed. Count: Crtl 1: 5 St 66. Crtl 2: 5 St 66. Crtl 3: 3 St 66. Exp 1: 5 St 66. Exp 2: 3 St 66. Exp 3: 5 St 66. All active. Exp 3s most hyper.

11/14/2024

Changed and treated water. Fed. Count: Crtl 1: 5 St 66. Crtl 2: 5 St 66. Crtl 3: 3 St 66. Exp 1: 5 St 66. Exp 2: 3 St 66. Exp 3: 5 St 66. All very active. Exps (especially Exp 3, seem to spend more time at the surface of the water.

11/16/2024

Tanks cleaned and treated, and frogs fed by Dr. Crain. All seem healthy. There was not enough dechlorinated water for tank 6, so more dechlorinated water was made.

11/18/2024

Changed and treated the water. All frogs are healthy and active. More Exp 3s swimming to the surface than in other tanks. Crtl only had one frog at the surface.

11/20/2024

Starting with Ctrl 1 tank, placed all frogs in a watch filled with MS-222 (tricaine methanesulfonate) to anesthetise them. The solution is made of 2g/L MS-222 + 1.5 g $NaHCO_3$(sodium bicarbonate). Each frog was then removed from the solution, placed on a weigh boat on a scale, and weighed. Crtl 1 weights were: Frog 1: 1.08 g, Frog 2: 0.84 g, Frog 3: 1.28 g, Frog 4: 0.98 g. Frog 5: 0.97 g. Each frog (still on the weigh boat) was placed under a dissection microscope, and dissected at the abdominal cavity. The liver was removed, placed in a bullet tube, and fixed in Bouin's solution. The tube was labeled as follows: C_1= Crtl 1, 1 = Frog 1, L = liver.

The above steps were repeated for each frog, and the bullet tubes were labeled accordingly. E_1etc. was used for the Exps. The weights were: Crtl 2 Frog 1: 0.70g, Frog 2: 1.52 g, Frog 3: 1.16 g, Frog 4: 1.36 g, Frog 5: 1.04 g. Crtl 3 Frog 1: 1.81 g, Frog 2: 1.06 g, Frog 3: 0.73 g. Exp 1 Frog 1: 1.07 g, Frog 2: 0.82 g. Frog 3: 0.94 g, Frog 4: 0.67 g, Frog 5: 0.82 g. Exp 2 Frog 1: 2.05 g, Frog 2: 1.06 g, Frog 3: 0.94 g.. Exp 3 Frog 1: 1.64 g, Frog 2: 1.31 g, Frog 3: 1.19 g, Frog 4: 0.77 g, Frog 5: 0.63 g.

Exp 1 Frog 2 showed underdeveloped limbs compared to Crtls. The intestine was also smaller, and had a darker colour (dark pink/grey).

Clearing

12/2/2024

The Bouin's was poured out into an old jug under the hood vent and the jug was sealed. We used 70% ethanol to clear tissues of Bouin's solution. The ethanol & old Bouin's

solution was poured into an old jug, which was then sealed. Samples were left in the ethanol for 4 weeks (over Christmas break).

1/14/25 – 1/21/2025

To continue to clear the fixative from the tissues, we repeated the clearing steps above daily until the solution was nearly clear

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