HISTOLOGICAL ANALYSIS OF DICLOFENAC'S EFFECTS ON LIVER IN *XENOPUS LAEVIS* METAMORPHS

A Report of a Senior Study

by

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ABSTRACT

Pharmaceuticals contamination in aquatic ecosystems poses increasing threats to wildlife health, with compounds such as diclofenac frequently detected in surface waters due to incomplete removal during wastewater treatment. This study examined the effects of 21-day exposure of diclofenac on the liver of *Xenopus laevis* metamorphs. Tadpoles were divided evenly into control and experimental (0.125μg/L diclofenac) groups, housed in six tanks containing 10 individuals each and 4 L of dechlorinated water. Following exposure, liver tissues were fixed, sectioned, and stained for histological analysis. Hepatocyte size showed no significant difference between groups, with mean lengths of 20.57 ± 2.20 μm for controls and 20.64 ± 2.17 μm for treated frogs (p-value = 0.667), and widths of 20.66 ± 9.48 μm and 20.84 ± 8.59 μm (p-value = 0.723). However, the number of Von Kupffer cells was significantly higher in the diclofenac group (61.9 ± 8.40 cells per field) compared to controls (35.13 ± 6.53 cells per field; $p < 0.0001$), indicating hepatic immune activation. These findings suggest that diclofenac may trigger sublethal liver inflammation even without structural hepatocyte damage. Future research should extend exposure duration, use adult *X. laevis*, and increased concentration to better understand pharmaceutical effects on amphibian liver function and ecotoxicology.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the Maryville College Biology Department for funding this study. I am sincerely thankful to Dr. Crain for his guidance and support throughout this project, as well as to my lab partner, Emily Miller, for her collaboration. I also wish to thank Emma Belica, Lily Petree, and Jacob Berven for their continual encouragement during the course of this study. Lastly, I am deeply grateful to my family and friends for their unwavering support and for helping me pursue my goals and dreams.

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CHAPTER I

INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are essential for human health but have become a significant environmental concern due to their presence in aquatic ecosystem. These compounds enter water bodies primarily through wastewater effluents, where they can affect non-target organisms. Among pharmaceutical contaminants, diclofenac and triclosan are two studied compounds due to their toxicity to aquatic species, including amphibians. Both compounds have been detected in surface waters and are known to cause physiological and histopathological damage to aquatic organisms (Cunningham et al., 2006).

As the use of pharmaceuticals and personal care products continues to rise, their active ingredients, whether in their original form or as metabolites, enter the environment through various pathways. Many of these compounds persist in trace yet measurable amount, raising concerns about their long-term impact on the aquatic ecosystems.

In addition to human excretion, active pharmaceuticals ingredients (APIs) can be introduced into ecosystems through agriculture use, livestock waste, and pharmaceuticals manufacturing processes (Cunningham et al., 2006). Pharmaceuticals have been detected in aquatic environments worldwide, primarily due to their widespread use and the insufficient removal efficiency of sewage treatment plants, or in some cases, the complete absence of such treatment facilities. Pharmaceutical concentrations in surface waters typically fall within

the range of low nanograms per liter (ng/L) to low micrograms per liter (μg/L). These levels are influenced by factors such as population density within the watershed, the capacity of the receiving water body, and the efficiency of wastewater treatment technologies. However, localized contamination from specific point sources, such as pharmaceutical manufacturing facilities, can lead to significantly higher concentrations, reaching milligrams per liter (mg/L) in affected surface waters (Brodin et al., 2014).

Many pharmaceuticals are formulated to act rapidly within the human body and are excreted largely intact, allowing them to enter freshwater systems in an active form. Certain pharmaceutical compounds, including antidepressants, selective serotonin reuptake inhibitors (SSRIs), hormones, antihistamines, and other psychiatric medications, have been shown to influence behavior in aquatic animals. Even at environmentally relevant concentrations, these substances can induce sub-lethal effects in aquatic organisms, raising concerns about their long-term ecological impact (Brodin et al., 2014). The accumulation of these pharmaceuticals in aquatic environments not only affects individual species but may also disrupt predator-prey interactions, reproductive success, and overall ecosystem stability.

Various other drugs, including antiviral, thyroids, anti-inflammatory, and antidepressants, have also been shown to interfere with development, behavior and physiology. These contaminants, often introduced through wastewater effluents, can alter hormone regulation, disrupt normal growth patterns, and impair neurological function. Table 1 provides an overview of key pharmaceuticals pollutants and documented effects on the model experimental amphibian *Xenopus laevis*.

Table 1. The Effects of Various Drugs on *Xenopus laevis.*

Source	Drug	Type of Drug	Dose	Key findings
Fogliano et al., 2023	Delorazepam	Psychotropic	1 µg/L	Decreasing heart rate and motility, induces cephalic and abdominal edema, increases (reactive oxygen species) ROS production,
			5 µg/L	
			10 µg/L	
Laçin et al., 2024	Favipiravir and Oseltamivir	Antiviral	137.9 mg/L	Reduced biochemical enzymes activities in embryos, altered biochemical responses
			32.3 mg/L	
Boran et al., 2021	Levothyroxine	Thyroid	0.001 mg/L	Progression in developmental stage, increase in thyroxine level in 7 days, increase in lactate dehydrogenase, impacted energy metabolism
			0.0001 mg/L	
	Propylthiouracil	Antithyroid	0.1 mg/L	
			1 mg/L	
Cardoso et al., 2017	Diclofenac	Anti-inflammatory	1 mg/L	Toxic during development, toxicity the pharmaceutical products, liver and kidney damage
			4 mg/L	
			8 mg/L	
			16 mg/L	
			32 mg/L	
			62.5 mg/L	
Conners et al., 2009	Fluoxetine	Selective serotonin reuptake inhibitors (SSRIs)	0.1 µL	No effects
			1 µL	
			10 µL	Reduced growth at metamorphosis
	Sertraline		0.1 µL	Acceleration of development
			1 µL	
			10 µL	

Continuation of Table 1.

Johnson et al., 2024	Atorvastatin	Statin		Dysregulation of the cholesterol biosynthesis pathways, altering expression of genes
Blahova et al., 2021	Fluoxetine	Antidepressant	0.1; 1; 10; 100; 1000; 10,000 μg/L	Increases heart rates and hatching rates
	Citalopram		0.01; 0.1; 1; 10; 100; 1000; 10,000; 100,000 μg/L	
Islas-Flores et al., 2019	Naproxen	Non-steroidal anti-inflammatory	0.12 and 2.30 μg/L	Malformations, inhibition of growth, mortality observed
Pablos et al., 2020	Carbamazepine	Analgesic		Effects the endocrine system
Fogliano et al., 2023	Delorazepam	Psychotropic	1 μg/L	Increase in genomic DNA methylation, unbalancing apoptosis/ proliferation pathways, aberrant expression of DNA-repair genes
			5 μg/L	
			10 μg/L	

Triclosan

Among these contaminants, one pharmaceutical contaminant of particular concern is triclosan, an antimicrobial agent widely used in personal care products. Due to its persistence in aquatic environments, triclosan has been studied for its toxic effects on amphibians, including *Xenopus laevis.* Research has demonstrated that triclosan exposure can lead to

significant physiological and histopathological alterations, even at low concentrations. Table 2 summarizes studies investigating the dosage-dependent effects of triclosan on *X. laevis* and highlights the severity of its impact on amphibian health.

While triclosan's environmental persistence and toxicity have raised concerns, another pharmaceutical contaminant of significant interest is diclofenac, a widely used non-steroidal anti-inflammatory drugs (NSAID). Unlike triclosan, which is primarily an antimicrobial, diclofenac is a pain reliver and anti-inflammatory agent that has become one of the most detected pharmaceuticals in aquatic environments. Its widespread use and poor biodegradability contribute to its accumulation in water bodies, where it has been linked to toxic effects in aquatic organisms, particularly amphibians.

Diclofenac

Advancement in pharmaceutical sciences particularly in formulating medications into physical dosage forms like tablets and capsules have significantly enhanced drug performance. Identifying the mechanism of action behind non-steroidal anti-inflammatory drugs (NSAIDs) led to the creation of numerous new medications, including propionic acid derivatives such as ibuprofen and fenamic acid (Altman et al., 2015). Diclofenac, one of the most widely NSAIDs worldwide, has played a critical role in medicine since its development in the 1973 (Altman et al., 2015). Diclofenac (DCF) is classified as an NSAID that functions by inhabiting both COX-1 and COX-2 enzymes (Altman et al., 2015). Like other NSAIDs, diclofenac usage carries a heightened risk of severe gastrointestinal, cardiovascular, and renal side effects, particularly at higher doses (Altman et al., 2015).

Table 2. Overview of Triclosan Dosage and Its Effects in *Xenopus laevis* Studies.

Study	Administration Method	Dose	Key findings
Tenkov et al., 2022	In water, Diluted with ethanol	0.5 mg/L	Alive throughout the experiment; osmotic resistance decreased; significant liver damage
		1 mg/L	Death on day 11th; increased areas of hemorrhage and higher number of melanomacrophage
		2 mg/L	100% mortality on the 4th day
Fort et al., 2010	Abiotic water exposure	0.2 µg/L (control)	No mortality observed; thyroid-mediated metamorphosis
		0.6 µg/L	
		1.5 µg/L	
		7.2 µg/L	
		32.3 µg/L	2.5% Mortality observed
Matsumura et al., 2005	Water exposure	20 µg/L	No mortality observed; lower plasma Vg levels; lower plasma T levels
		100 µg/L	
		200 µg/L	
	Intraperitoneal Injection	4 µg/g body weight	
		40 µg/g body weight	
		400 µg/g body weight	
Thompson et al.,	Water exposure	0.1 µM	Above 5 µM lead to mortality
		0.5 µM	
		1 µM	
		5 µM	
		10 µM	
		50 µM	
		30 µM	Alive throughout the experiments; impair mitochondrial structure; disrupting cellular and molecular pathways
		10 µM	
		1 µM	
Babalola et al.,	Water Exposure	0.5 mg/L	Delayed tadpole development, reduced hind-limb length, increased the whole-body mass

Upon oral administration, diclofenac is rapidly absorbed into the bloodstream, and its absorption levels increase proportionally with the administered dose. However, the absorption rate can vary based on factors such as its salt formulation, pharmaceutical preparation, and whether it is taken with or without food. Due to its short biological half-life and rapid elimination from the body, diclofenac usually requires frequent dosing to maintain effective therapeutic concentrations, potentially elevating the risk of adverse effects. Clinical studies have repeatedly demonstrated the efficacy of enteric-coated diclofenac sodium tablets in alleviating pain and inflammation in conditions such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gout (Altman et al., 2015).

Diclofenac is frequently detected in various water bodies, including rivers, lakes, and coastal areas. Its presence is primarily attributes to human pharmaceutical use, with the compound entering aquatic environments through wastewater effluents (Bonnefille et al., 2018). Diclofenac concentrations vary significantly across different aquatic environments. Concentrations in marine ecosystems have been reported within a broad range, from 0.000001 μg/L to 0.843μg/L (Bonnefille et al., 2018). It has adversely affected a range of marine species and notable effects include histopathological changes in fish, such as gills and liver damage, even at low concentration. While diclofenac does not significantly bioaccumulate in aquatic organisms, its continuous input and persistence raise concerns about chronic exposure and long-term ecological consequences (Bonnefille et al., 2018).

Additionally, diclofenac is known for its resistance to biodegradation, resulting in its limited removal during standard biological wastewater treatment processes (Vieno et al., 2014). Advanced wastewater treatment methods, including membrane-based or specialized biological systems, have shown greater effectiveness in eliminating diclofenac compared to

conventional activated sludge processes. Extending the treatment duration, particularly to retention times exceeding two to three days, can also markedly enhance the removal efficiency of diclofenac during biological treatment (Vieno et al., 2014).

Given its widespread presence in aquatic environments, various studies have investigated the effects on diclofenac at different concentrations on amphibians. Research has demonstrated that even at low doses, diclofenac can cause significant physiological and histopathological changes, particularly in the liver and kidneys. Table 3 summarized key studies examining the dosage-dependent effects of diclofenac on *Xenopus laevis* and other species, highlighting its impact on amphibian health.

Model Organism

To better understand the physiological impact of diclofenac on amphibians, an appropriate model organism is required. Model organisms are essential in biological research, providing invaluable insights into genetics, development, and disease that would be difficult to study in humans or more complex systems. One such model organism that has significantly contributed to scientific research is *Xenopus laevis*, an amphibian widely used in molecular biology (Cannatella et al., 1993). *Xenopus* serves as an ideal laboratory model due to its ease of breeding and maintenance in controlled environments. They are commonly referred to as the African clawed frog, known for its distinctive webbed hind feet (Cannatella et al., 1993 and Carotenuto et al., 2023). The natural range of *X. laevis* spans a vast portion of Sub-Saharan Africa, extending from the Republic of South Africa northward to Zaire and Uganda, and westward to Cameroon, with a preference for cooler upland habitats (Cannatella et al., 1993).

Table 3. Overview of Diclofenac Dosage and Its Effects in *Xenopus laevis* Studies.

Study	Administration Method	Dose	Key findings
Peltzer et al., 2019	Waterborne Acute exposure (96h test)	125-4000 μg/L	Increased mortality at higher doses at 2462.29 μg/L and 2828.43 μg/L
	Waterborne Chronic exposure (20-22 days)	125-2000 μg/L	Delayed larval development, growth inhibition, reduced body condition
	Waterborne Chronic exposure (sublethal effects)	125-2000 μg/L	Abnormalities in body axis, craniofacial structures, gut malformations, and organ damage (microcardia, cholecystitis)
	Waterborne Chronic exposure (cardiotoxicity effects)	1000-2000 μg/L	Significant reduction in heart rate and ventricular systole intervals, indicating impaired cardiac function
	Waterborne Chronic exposure (neurotoxicity effects)	125-2000 μg/L	AChE activity was initially inhibited but increased at higher concentrations, suggesting neurological disruption
	Waterborne Chronic exposure (swimming behavior effects)	125-250 μg/L	Decreased swimming activity, reduced movement. And velocity at lower doses
	Waterborne Chronic exposure (hyperactivity at high doses)	1000-2000 μg/L	Increased swimming activity at high doses, indicative of stress response

Continuation of Table 3.

Cardoso et al., 2017	Waterborne exposure (96h – acute toxicity)	1-62.5 mg/L	Dose-dependent mortality and malformations with 9.56mg/L in *X. laevis*
	Waterborne exposure (96h – teratogenic effects)		Malformations included axial deformities (bent notochord, short tail), edema (craniofacial, cardiac, abdominal), and stunted growth, higher doses lef to 100% mortality
	Waterborne exposure (Growth inhibition analysis)		Significant reduction in embryo length with increasing diclofenac concentrations
Reis et al., 2024	Waterborne teratogenesis assay	1 mg/L	Induced axial malformations, edema, and growth inhibition; causes oxidative stress, thyroid-disrupting effects
		4 mg/L	
		8 mg/L	
		16 mg/L	
		32 mg/L	
		62.5 mg/L	

One of the key advantages of *X. laevis* is its evolutionary similarities to higher vertebrates, particularly in terms of physiology, gene expression, and organ development including the eyes, liver, lungs, heart, and kidneys which enables the direct application of research findings to human biology. *X. laevis* has progressively drawn the interest of ecotoxicologist and has been utilized in various ecotoxicological studies. Due to its external fertilization, development, and highly permeable skin, it is highly sensitive to environmental pollutants, making it a reliable indicator of habitat diversity, biodiversity, and local environmental stressors. The direct exposure to contaminants in both water and air allows it

to effectively reflect levels of environmental pollution (Carotenuto et al., 2023). *X. laevis* has become an important key model for studying aquatic life and how it is affected by various environmental contaminants, making it invaluable for understanding the ecological impact of pollution on amphibians and other aquatic organisms.

Among the various physiological systems affected by environmental contaminants, the liver and kidney play a crucial role in mediating toxicity and regulating the organism's response to pollutants. Studying the impact of Diclofenac on these organs in *X. laevis* provides valuable insight into how pollution influences amphibian health and survival. The liver in amphibians, including *X. laevis*, functions similarly to that of other vertebrates, including mammal (Crawshaw et al., 2000). The liver is composed of two distinct lobes, the left and right, which function independently. Hepatocytes in the liver are organized in clusters and cords, interwoven with a network of sinusoids (Crawshaw et al., 2000).

The amphibian liver shares many physiological functions with that of other vertebrates, including roles in energy and protein metabolism, urea synthesis, bile salt excretion, biotransformation, and detoxification. In temperate species, the liver serves as a crucial energy reserve, accumulating glycogen and fat during autumn to sustain the animal through hibernation. Additionally, in female amphibians, the liver is responsible for producing vitellogenin, a protein-lipid precursor essential for yolk formation in developing eggs (Crawshaw et al., 2000). It is responsible for metabolic processes, including the ornithine (urea) cycle, an enzymatic pathway responsible for converting toxic ammonia into urea for safe excretion. Ammonia, a byproduct of protein metabolism, must be eliminated or detoxified to prevent harmful effects on the organisms (Crawshaw et al., 2000).

Beyond its metabolic and detoxification functions, the liver also serves an important immune role through the activity of Von Kupffer cells. Von Kupffer cells are special immune cells found in the liver that help remove harmful substances and damaged cells from the blood. They are part of the body's defense system and are located along the walls of the liver sinusoids. These cells can change their activity depending on what is happening in the liver. When the liver experiences stress or injury, such as from toxin, drugs, or infection, Kupffer cells become more active and may increase in number. They release chemical signals that help fight infection and clean up damaged tissue, playing an important role in how the liver responds to inflammation and begins to repair itself (Dixon et al., 2013). Figure 1 shows the structure and layout of the liver in adult *X. laevis*.

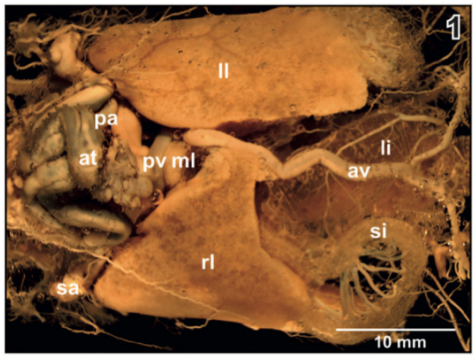

Figure 1. Liver of adult *Xenopus laevis*. This image shows the external morphology of Xenopus laevis, highlighting its multi-lobed structure (Lametschwandtner, Alois et al., 2022, pg. 4).

While the liver is important, the kidney plays a crucial role in excreting waste products and water regulation. The three different kidney types are pronephros, mesonephros, and metanephros. Although they differ in their overall structure and level of complexity, they all rely on nephrons as the essential units responsible for filtration and excretion (Mobjerg et al., 2000 and Droz et al., 2017). In amphibians, the pronephros functions during the early larval stages but degenerates as metamorphosis progresses. The mesonephros, however, remains and serves as the primary kidney in adult amphibians (Mobjerg et al., 2000). In adult

anamniotes, the mesonephros remains the primary excretory organ and is referred to as the opisthonephros (Droz et al., 2017). However, in amniotes, the mesonephros is only functional for a short period during embryonic development, lasting from a few days to several weeks depending on the species. As embryos mature, the mesonephros regresses while the metanephric kidney forms in a more posterior position. Containing up to a million nephrons per kidney, the metanephros becomes the permanent excretory organ in adult amniotes (Droz et al., 2017). Figure 2 displays the kidney structure and arrangement in adult *X. laevis.*

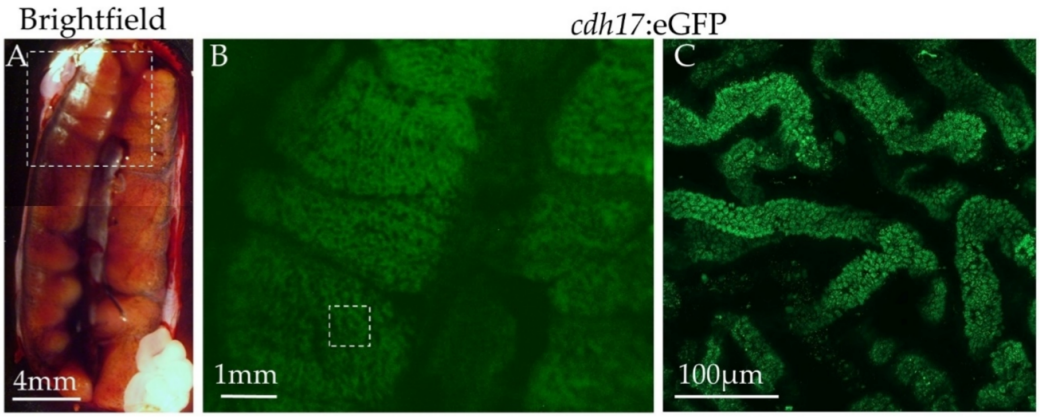

Figure 2. Kidney of adult *Xenopus laevis*. This image shows the elongated, darkly pigmented kidneys positioned dorsally along the vertebral column (Corkins et al., 2018, pg. 10).

Purpose of the Study

The purpose of this study is to investigate the effects of environmental exposure to diclofenac on the liver of *Xenopus laevis*. By simulating concentration that reflect those detected in natural aquatic ecosystems, this study aims to assess how this pharmaceutical contaminant impacts critical physiological organs in aquatic species. Understanding these effects will provide relevant data into the broader implications of pharmaceutical pollution on aquatic wildlife health.

CHAPTER II

MATERIALS AND METHODS

Xenopus laevis tadpoles were obtained from Xenopus Express (xenopus.com) and acclimated in dechlorinated water at room temperature. The water was prepared 24 hours in advance to ensure temperature stabilization and was treated with a water conditioner to remove chlorine. Tadpoles were then divided into three development stages (stages 63, 64, and 65) using *The Normal Table of Xenopus laevis* (Nieuwkoop and Faber, 1967). For each stage, animals were split evenly into two groups (control and experimental), housed in separate tanks containing 10 tadpoles each. In total, six tanks were set up, with each tank containing 4 L of dechlorinated water. During the process, tadpoles were fed frog food ad libitum and the amount consumed was not measured. In addition, ¼ of the water in the tanks was replaced every day. Xenopus were weighed before the start of the experiment and again at the end of 21-day exposure period to record any changes in body weight. All procedures were approved by the Maryville College IACUC committee (see Appendix 1).

Diclofenac was obtained from MedChemExpress (medchemexpress.com) and diluted in dimethyl sulfoxide (DMSO) to prepare the stock solution. Specifically, 0.005g of diclofenac was dissolved in 100mL of DMSO, creating a stock concentration of 50μL/mL. For the experimental groups, 10 μL of pure DMSO was added to the control tanks to

maintain consistency, giving an exposure concentration of 0.125µg/L. During each water change, 2.5 µL of the stock solution was reintroduced to the experimental tanks to ensure continuous drug exposure. The experiment lasted for 21 days, after which the frogs were anesthetized in MS222, and liver were removed using microdissection (Figure 3) and stained with Bouin's fixative. After clearing with 75% ethanol, histological analyses were performed on the liver from both control and experimental groups. The tissue was initially fixed in 70% ethanol, followed by embedding, sectioning, staining, and analyzed according to the procedure described in *Animal Tissue Techniques* (Presnell and Schreibman 1977).

Measurements of hepatocyte length and width, along with counts of Von Kupffer cells, were recorded within the field of view under 40x magnification. For each frog liver, twenty hepatocytes' measurements were taken to determine mean cell dimensions (Figure 4), and Von Kupffer cells were counted within the same field of view (Figure 5). Mean values and standard deviations were determined for each variable, and statistical significance between control and experimental groups was assessed using an unpaired t-test in MSExcel.

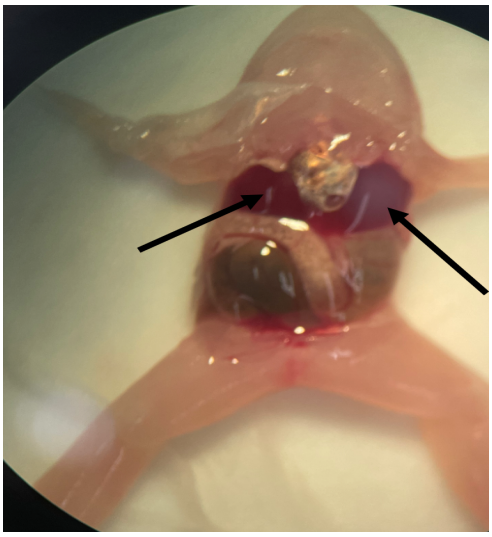

Figure 3. Liver placement in *Xenopus laevis* as viewed under a dissection microscope. The arrow points to the liver within the body cavity, illustrating its orientation and location.

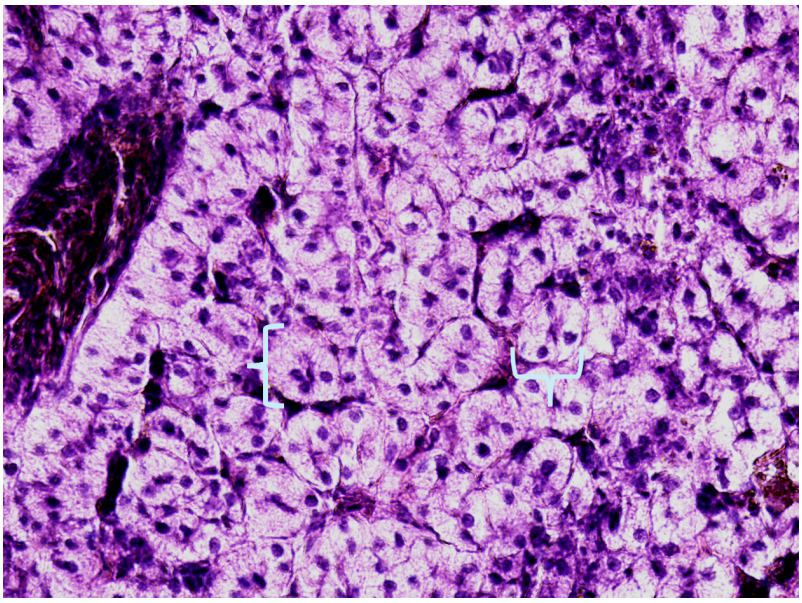

Figure 4. Control liver samples from *Xenopus laevis*. The two bracketed regions indicate where hepatocyte length and width were measured for comparison between the samples. Image captured under 40x magnification.

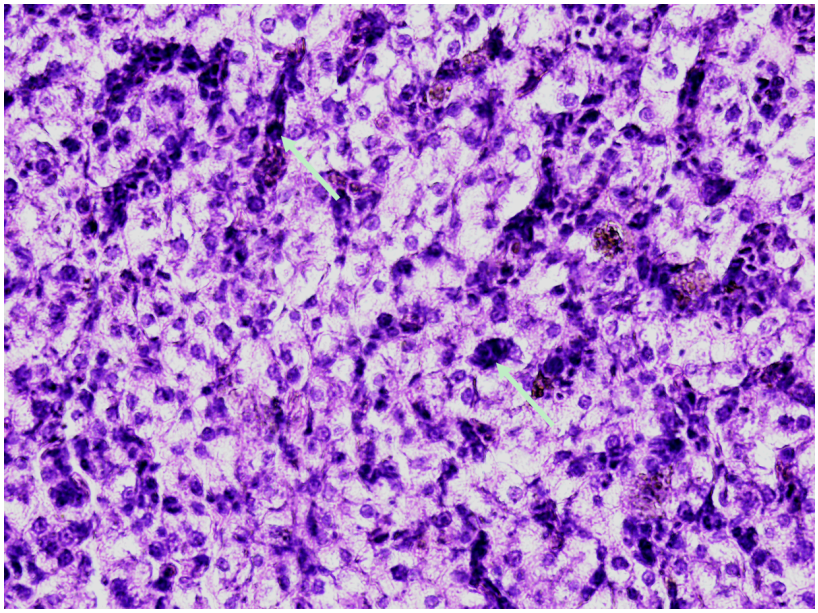

Figure 5. Liver section from experimental stage 65 *Xenopus laevis.* The arrow indicates the presence of Von Kupffer cells within the hepatic tissue. Image captured under 40x magnification.

CHAPTER III

RESULTS

After 21 days of diclofenac exposure, histological examination of liver tissue showed that hepatocyte size was similar between the control and experimental groups. The mean hepatocyte length for the control group was 20.57 ± 2.20 μm, and 20.64 ± 2.17 μm for the experimental group (p-value = 0.667; n = 30) (Figure 6). The mean hepatocyte width was 20.66 ± 9.48 μm in the control group and 20.84 ± 8.59 μm in the experimental group (p-value = 0.723; n= 30) (Figure 7).

In contrast, the number of Von Kupffer cells was significantly higher in the liver of diclofenac-exposed frogs compared to controls ($p<0.0001$; Figure 8). The experimental group displayed an average of 61.9 ± 8.40 cells per field, whereas the control group averaged 35.13 ± 6.53 cells per field of view. The number of Von Kupffer cells was compared between the control and experimental liver samples as shown in Figure 9. Body weight measurements taken before and after the 21-day period showed slight variation among individual frogs in both control and experimental groups. In the control group, frog 63 increased in weight from 1.724bg to 1.940 g, frog 64 decreased from 1.973 g to 1.746 g, and frog decreased from 1.820 g to 1.680 g, In the treated group, frog 63 increased from 1.380 g to 1.990 g, frog 64 showed a minor decrease from 1.800 g to 1.780 g, and frog 65 increased from 1.770 g to 1.880g.

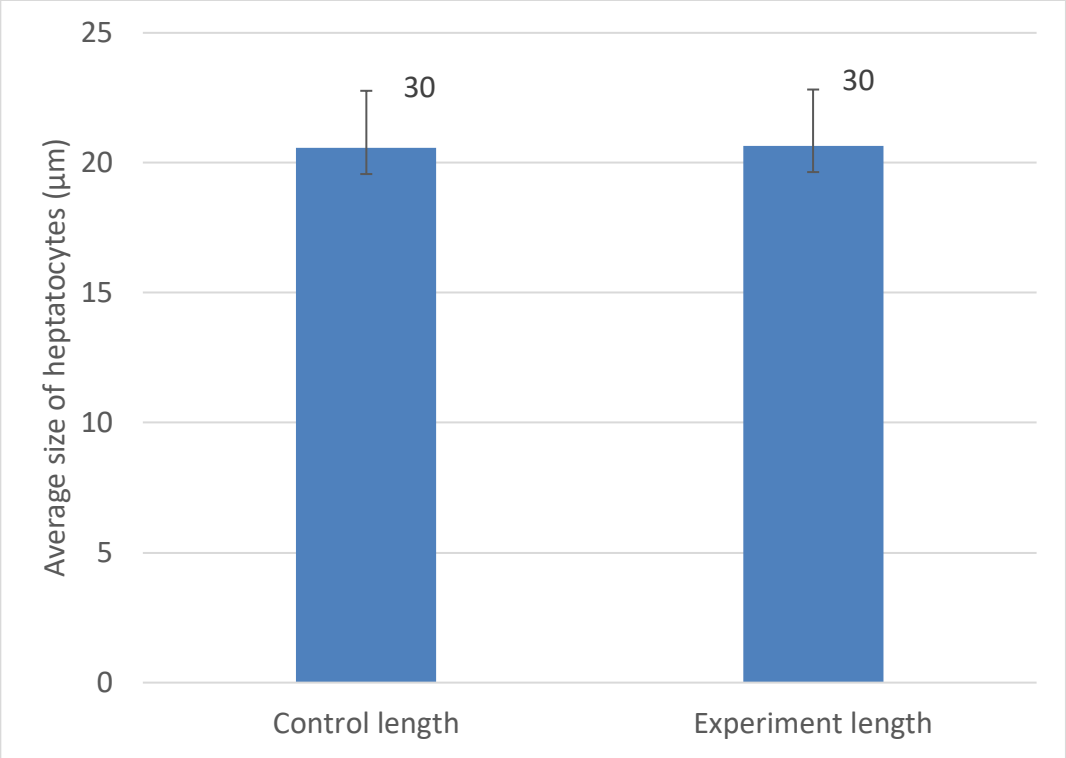

Figure 6. Average hepatocyte length (µm) in control and diclofenac-treated *Xenopus laevis* albino at 40x magnification. Bars represent mean ± SD for n = 30 measurements per group. No significant difference was observed between groups (p-value = 0.677)

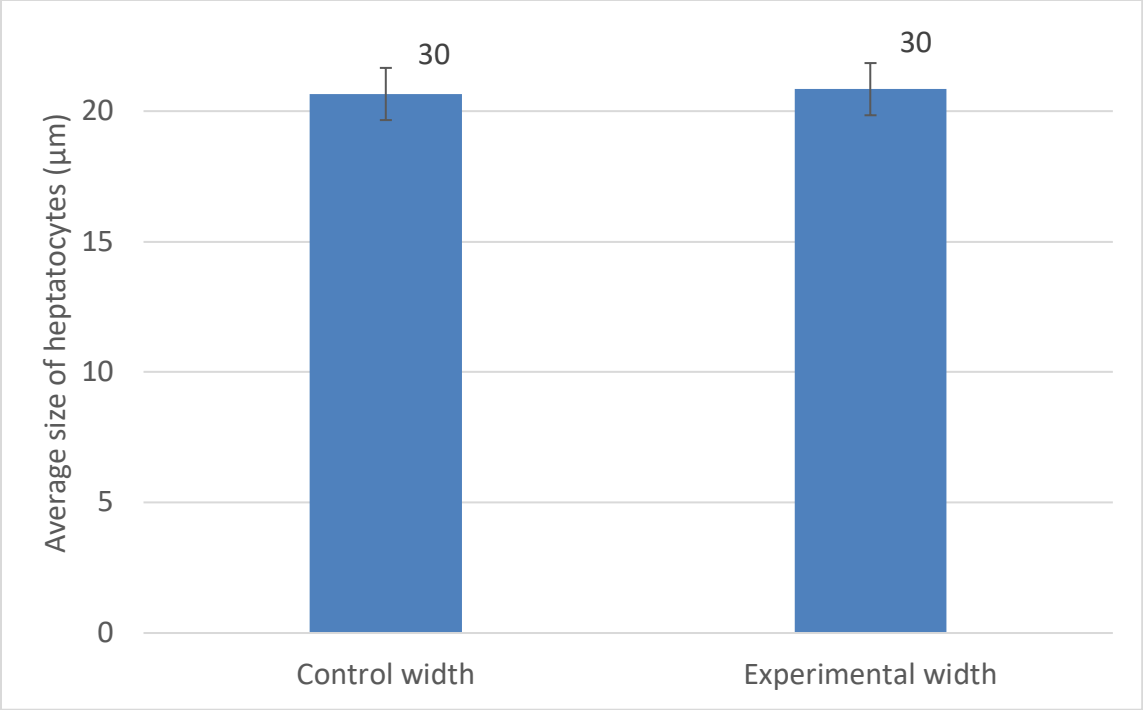

Figure 7. Average hepatocyte Width (µm) in control and diclofenac-treated *Xenopus laevis* albino at 40x magnification. Bars represent mean ± SD for n = 30 measurements per group. No significant difference was observed between groups (p-value = 0.723)

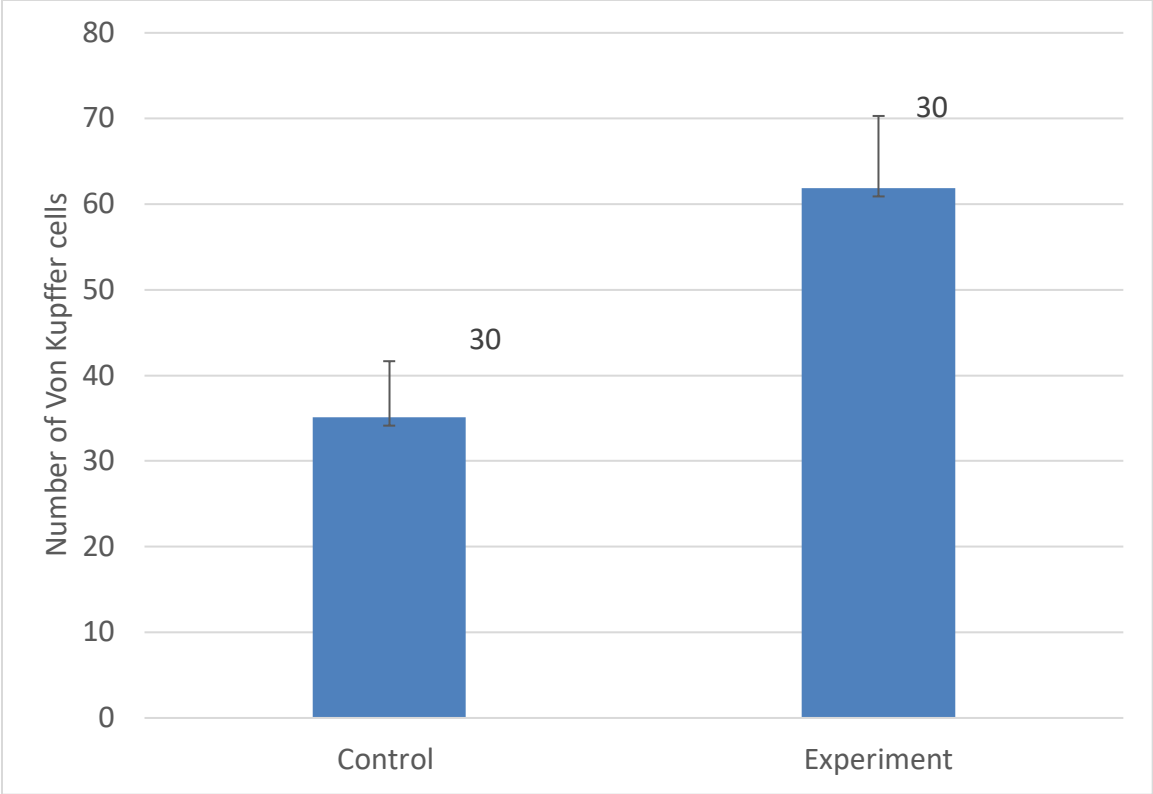

Figure 8. Number of Von Kupffer cells in liver tissue of control and diclofenac-treated *Xenopus laevis* albino at 40x magnification. Bars represent mean ± SD for n = 30 field of view. The difference between groups was statistically significant (p-value = 5.93×10^{-29}).

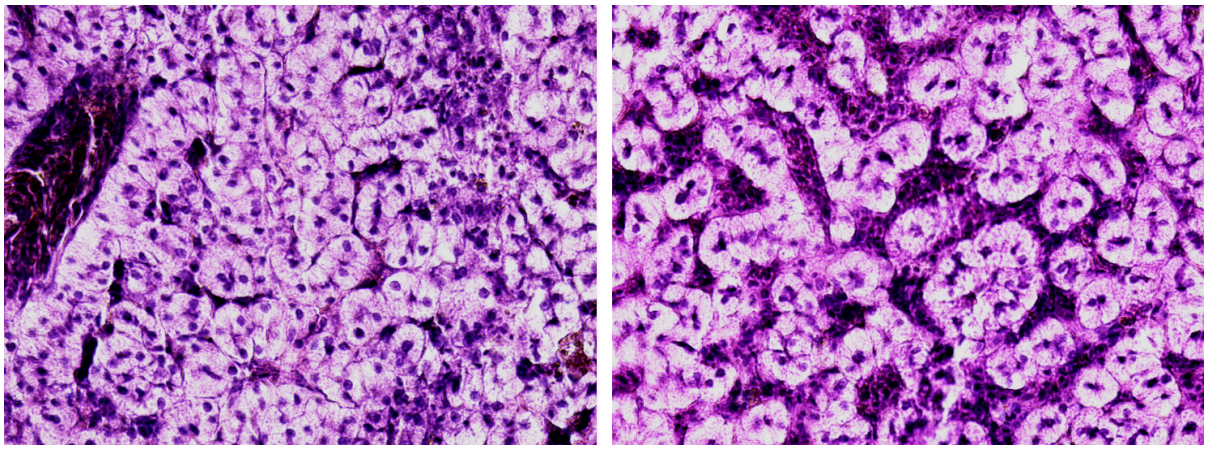

Figure 9. Comparison of Von Kupffer cells between control and experimental liver samples. The left panel shows the control sample, and the right panel shows the experimental sample.

CHAPTER IV

DISCUSSION

The results showed that 21 days of diclofenac exposure did not noticeably affect hepatocyte length or width in the experimental frogs compared to controls. However, the number of Von Kupffer cells was significantly higher in the diclofenac treated group. This finding suggests that while diclofenac did not cause measurable changes in hepatocyte size, it may have activate the liver's immune defense system. Von Kupffer cells are specialized macrophages found in the lives that help remove toxins, pathogens, and damages cells from circulation. An increased number of these cells reflects a heightened immune or inflammatory response to chemical exposure or stress in the liver. When the liver is exposed to chemical stressors, these cells become activated and may proliferate or recruit additional macrophages to help maintain homeostasis (Dixon et al., 2013).

Although similar responses have been reported in other research, most previous studies were conducted in different animal models and used toxicants other than diclofenac. One study found that Von Kupffer cells play a key role in mediating hepatic toxicity and carcinogenesis, releasing cytokines and reactive oxygen species that promote inflammation and tissue remodeling in response to chemical stressors (Roberts et al., 2007). Likewise, further study demonstrated that Von Kupffer cells provide a protective function during acetaminophen-induced liver injury in mice by clearing necrotic tissue and reducing further

damage (Ju et al., 2002). Similarly, Von Kupffer cells aid liver regeneration in mice following acetaminophen overdose by inducing the chemokine receptor on hepatocytes, which supports tissue repair (Nguyen et al., 2021). Although these studies were performed in mammals and with different toxicants, the observed increase in Von Kupffer cells in the present study is consistent with the general hepatic response seen across species when the liver encounters xenobiotics or environmental stressors.

The increased Von Kupffer cell count following diclofenac exposure may indicate that even low or sublethal doses of pharmaceuticals contaminants can activate immune mechanisms in aquatic organisms. Previous research has shown that chronic exposure to pharmaceuticals residues, including diclofenac, can cause hepatic changes in fish and amphibians (Bonnefille et al., 2018). The rise in Kupffer cells activity observed in this study could therefore represent an early immune reaction to pharmaceutical pollutants in the aquatic environment, signaling the liver's attempt to detoxify and protect itself from accumulating xenobiotics.

Future research should build upon these findings by extending the exposure period beyond 21 days to better assess chronic effects of diclofenac on liver structure and immune function. Conduction similar experiments on fully developed adult frogs may provide a more accurate representation of hepatic immune responses in mature organisms. Varying the diclofenac concentration to include higher exposure levels could also help identify potential dose-dependent effects on hepatocyte morphology and Kupffer cell activity. Combining histological analysis with biochemical assays would clarify whether the observed increase in Kupffer cells represent a protective adaptation or a sign of early toxicity. Expanding this

research could contribute to a deeper understanding of how pharmaceutical contaminants in aquatic environments affect amphibian health and liver physiology over time.

APPENDIX 1: IACUC approval

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

Provide information after each bold item
Student Name:
Diya Patel and Emily Miller
Student Email Address:
diya.patel@my.maryvillecollege.edu; emily.g.miller@my.maryvillecollege.edu
Date:
April 4, 2025
Senior Study Advisor:
Crain
Species to be used:
Xenopus laevis
Age of animals:
Stage 63-66
Number of animals in study:
30
Duration of study:
August 20-September 20
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.
Drew Crain	8652928737	86520287 37	

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.
Euthanasia via MS222

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

Maryville College IACUC Approval Number: 202502
Date Approved: Apr. 15, 2025
Signed:

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