THE EFFECTS OF EPIGALLOCATECHIN-3-GALLATE ON LIVER HISTOLOGY OF GOLDFISH (*CARASSIUS AURATUS*) AND AFRICAN CLAWED FROG TADPOLES (*XENOPUS LAEVIS*)

A Report of a Senior Study

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ABSTRACT

The use of supplements that contain green tea extract have drastically increased in recent years. A specific component of green tea extract, a catechin called (-) epigallocatechin-3-gallate (EGCG) is suspected to cause liver damage and toxicity at high doses. It was hypothesized that EGCG would cause damage to the hepatocytes of goldfish (*Carassius auratus*) and African clawed frogs (*Xenopus laevis*). During this experiment, five goldfish and five African clawed frogs where exposed to 100µg/L (-) epigallocatechin-3-gallate (EGCG) for 14 days. Goldfish and tadpoles where observed daily and weighed every five days. The behavior of the tadpoles was unchanged while the goldfish exhibited strange behavior. Their livers where then removed, embedded, sectioned, and stained through the process of histology. The weight for the goldfish showed no significant difference between control or treated individuals, whereas tadpole weight from day 0-10 was near significance ($p=0.07$) with treated individuals losing more weight. Hepatocytes for the control goldfish had an average size of 7.7µm, whereas the average hepatocyte size of the EGCG exposed goldfish was 5.75 which suggests an effect. However, for *Xenopus laevis* tadpoles, there was no difference in hepatocyte size ($p=0.74$). Future studies should further characterize hepatocyte damage induced by high doses of EGCG.

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CHAPTER I

INTRODUCTION

Foods and Beverages that Induce Liver Damage

Living in the modern world has caused an increase in stress levels, poor eating habits, lack of exercise, and obesity (Kioukia-Fougia 2016). However, due to increasing health literacy, in recent decades, the general population in developed countries have sought dietary changes that are healthy (Pleasant 2015). One of these dietary changes has been the prevalence of nutritional supplements which try to combat nutrition depletion (Kioukia-Fougia 2016). Nutritional supplements are also claimed to assist in gaining or losing weight, improving cognitive skills, and even increasing sexual behavior (Kioukia-Fougia 2016). Because of this, the market for health supplements for weight loss and energy boosters is projected to increase drastically (Dietary Supplements Market Size 2018).

Because nutritional supplements are not prescribed by a medical professional and instead are bought over the counter and through the internet, they are more accessible to the general population (Kioukia-Fougia 2016). The vitamin supplement market earned a revenue of more than 50 billion dollars in 2015 within the United States, while the global dietary supplement market is expected to gross 278 billion dollars by 2024 (Dietary Supplements Market Size 2018). In addition to this, many people who take these supplements are trying to maintain a

healthy lifestyle on the nutritional level (Dietary Supplements Market Size 2018). What many people do not realize is that these health supplements are having negative effects on their body's health, in opposition to their advertised benefits.

The broad market and widely successful commercialization of these nutritional supplements is leading to possible public health risks (Kioukia-Fougia 2016). An example of this is how iron supplements are being researched more for their possible harmful effects on the human body (Kioukia-Fougia 2016). Other supplements like hydroxyl citric acid that have been removed from the market based on 23 reports that linked serious health problems like jaundice, elevated liver enzymes, that resulted in liver damage and liver transplant (Kioukia-Fougia 2016). Indeed, several common foods and beverages have been linked to liver disease and liver damage (Table 1).

Table 1: Various foods, beverages, drugs that have been linked to hepatoxicity due to the amounts consumed. Effects of these include liver cancer and damage in addition to damage of other organs.

Food/Beverage	Effects	Reference
Acetaminophen	Liver damage in addition to loss of appetite, vomiting and jaundice	(Center for Drug Evaluation and Research 2013)
Garlic	Toxic to the liver, heart, and kidneys	(Banerjee 2003)
Milk & Milk products containing Aflatoxins contamination	Toxic and carcinogenic to the liver	(Office of Regulatory Affairs 2015)
Hydroxyl Citric Acid	Banned in 2009 in the US by FDA due to jaundice elevated liver enzymes, required liver transplant	(Kioukia-Fougia 2016)
Red Bull	Sinusoidal Swelling of hepatocytes	(Salih 2018)
Green Tea	Jaundice, hepatoxicity resulting in liver transplant	(Younes 2018)

One beverage that has been suggested to be linked to liver disease is green tea. Tea has been exported to various countries since the 17th century and has become one of the most popular consumed beverages (Sachdev 2017). Green tea is produced by harvesting fresh leaves of *Camellia sinensis* and immediately steaming them to prevent fermentation prior to dehydration (Sachdev 2017). In contrast, black tea is fermented and contains more caffeine than green tea while aiding in different biological activities (Sachdev 2017). A positive connection has been made between human health and green tea since the beginning of its production (Sachdev 2017). Green tea lowers blood cholesterol, blood pressure, and aids in preventing cancer due to its composition of antioxidants and polyphenols (Nakamura 2016).

While green tea has been linked to helping prevent oncogenesis, green tea extract has been linked to cases and occurrences of liver disease (Younes 2018). These possible links are due to one or more of the components of green tea that found in weight loss supplements (Table 2). The components of green tea extract are mainly polyphenols, which are catechins, theaflavins, tannins, and flavonoids which can be seen in Figure 1 (Senanayake 2013). A specific catechin, (-) epigallocatechin-3-gallate (EGCG), is the most relevant catechin in green tea and the main catechin that has been linked to hepatoxicity (Younes 2018). The average daily intake of EGCG from the consumption of green tea ranges from 90-300mg/day (Younes 2018). However, health supplements containing the green tea catechin provides a range of 5-1000mg/day for adults (Younes 2018).

Table 2: The Principle Constituents in Green Tea Extract (from Graham 1992) and the function.

Components	**Components Green Tea (% weight of extract solids)**	**Function**	**Reference**
Catechins	30-42	Produce 30-40% of solid product from dried green tea leaves that have been shown to exhibit antioxidative, anticarcinogenic, anti-inflammatory, and have antimicrobial properties	Molinari 2006
Flavonols	5-10		
Other Flavonoids	2-4		
Theogallin	2-3	Derived from gallic acid and quinic acid	PubChem Compound Database
Other depsides	1		
Ascorbic Acid	1-2	Related to glucose, can be found naturally in citrus fruits and many vegetables, necessary in human body to maintain connective tissue and bone.	PubChem Compound Database
Gallic Acid	0.5	Used in photography, pharmaceuticals, and as an analytical agent	PubChem Compound Database
Quinic Acid	2	Found in cinchona bark and in other plants	PubChem Compound Database
Other Organic Acids	4-5		

Table 2 (Continued): The Principle Constituents in Green Tea Extract (from Graham 1992) and the function.

Components	**Components Green Tea (% weight of extract solids)**	**Function**	**Reference**
Theanine	4-6	Can be found in saliva, mushrooms, and the fungus Imleria badia	PubChem Compound Database
Other Amino Acids	4-6		
Methylxanthines	7-9	One of the major metabolites of caffeine in the human body	PubChem Compound Database
Carbohydrates	10-15	Major contributors of nutrition in the human body, help provide energy	Hall 2016
Minerals	6-8		
Volatiles	0.02	Part of a plant that attracts pollinators and seed dispersers, and is used in perfumes and flavor compounds	Wiley Online Library

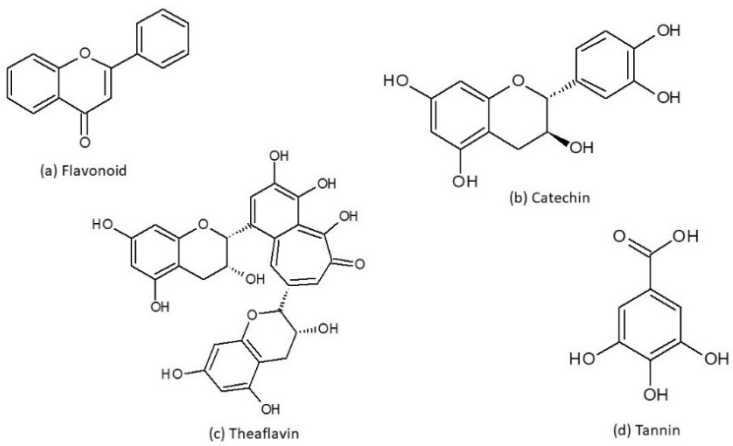


Figure 1: Chemical structures of the components of green tea extract. (a) Flavonoids (b) Catechins (c) Theaflavins (d) Tannins.

There have been many cases linking the effects of green tea extract to harmful effects on the liver. Excessive exposure to GTE has resulted in complete liver failure requiring liver transplant (Molinari 2006). Lower exposure levels also alter liver function, which can include hepatocellular necrosis as well as mixed inflammatory infiltrate (Molinari 2006). More specifically, the green tea extract caused coagulopathy and while also requiring a replacement of the central vein that resulted to the liver transplant (Molinari 2006). Many of the case studies on patients who have gotten hepatoxicity from green tea extract supplements experienced had elevated serum alanine levels, aminotransferase and bilirubin levels, as well as periportal and portal inflammation (Lambert 2007). There are other studies that suggest that the elevated levels of EGCG in green tea extract have led to hepatoxicity, nephrotoxicity, and intestinal toxicity in dogs, and suggest that toxicity in humans should be closely monitored because of this (Lambert 2007).

These polyphenols specifically have been linked to causing other effects in addition to liver damage. For example, the specific method of green tea preparation influences the amount of catechins contained within the product (Sachdev 2017), and catechins inhibit iron

absorption (Rani 2018). A low amount of iron within the body results in decreased production levels of hemoglobin, which can lead to anemia (Rani 2018). Some of the symptoms of anemia are the feeling of weakness, loss of concentration, and headaches (Rani 2018). While there is research that both suggests that there is no correlation between green tea and a lower hemoglobin level within the body, there have been other studies that state that the influence of green tea significantly decrease the hematocrit (Rani 2018).

Liver Structure

The liver is the largest metabolic organ within the human body that contributes to approximately 2% of total body weight in the average human adult (Hall 2016). In humans, the liver is an organ on the right side of the abdominal area above the stomach and below the diaphragm that approximately weighs about 3-3.5 pounds (Narins 2013). About 13% of the body's blood supply is held within the liver and circulated throughout the body by the portal vein and the hepatic artery (Narins 2013). The hepatic artery provides arterial blood to the gland while the portal vein carries blood from the esophagus and gastrointestinal tracts, from spleen and pancreas (Ross et al. 2003).

In addition to filtering and storing blood, the liver also regulates the metabolism of carbohydrates, proteins, fats, hormone and foreign chemicals, the formation of bile, the storage of vitamins and iron, and the formation of coagulation factors (Hall 2016). Having both arterial and venous blood supplies plus venous drainage makes the liver unusual (Young 2006). However, if the liver is impaired from doing any of these major functions including regulating blood clotting, metabolizing alcohol, and monitoring chemical and drug blood levels, this can lead to liver disease (Narins 2013).

Liver diseases can be classified as either acute or chronic. An acute liver disease or injury would be one that could be resolved within 6 months (O'Grady 2006). Examples of this type of injury could be caused by could include viral hepatitis or exposure to a toxin or medication (O'Grady 2006). A chronic liver disease would result in the liver being enlarged and firm (O'Grady 2006). Some examples of chronic liver disease are alcoholic liver disease and drug induced liver disease (O'Grady 2006). Symptoms including nausea, vomiting, loss of appetite, and jaundice, which is the yellowing of the eyes all are signs some type of liver disease (Center for Drug Evaluation and Research 2013). There have also been links between over-the-counter medications and herbal remedies that have been reported to cause liver abnormalities (O'Grady 2006).

The composition of the liver consists of hepatocytes, sinusoids, central and hepatic veins, and perisinusoidal space, Kupffer cells, and hepatic stellate cells. and the portal triad that consists of a venule branch, an arteriole branch, and small bile ducts (Table 3). These components of the liver are what is analyzed when trying to diagnose a liver disease like cirrhosis of the liver (Junqueira 2003). For liver cirrhosis, there is fibrosis and proliferation of fibroblasts and hepatic stellate cells that disrupt the normal liver function (Junqueira 2003). This means that the liver cells are separated from normal sinusoidal blood flow and have a reduced function like not being able to synthesize albumin or reducing the secretion of bile (Young 2006). The different types of irrigation as well as hepatocytes and other liver components can be seen in Figure 2.

Table 3: Components of normal liver and its function.

Component	Function	Reference
Hepatocytes	Has an exocrine function for the secretion of bile components. Process contents of the blood including major plasma proteins.	(Junqueira 2003)
Sinusoids	Blood flows through these from the hepatic portal veins and hepatic artery.	(Young 2006)
Central Veins	Vein that carries blood to the hepatic portal vein.	(Junqueira 2003)
Hepatic Veins	Veins that empty into the inferior vena cava.	(Junqueira 2003)
Kupffer Cells	Detect and phagocytose aged erythrocytes which helps free heme and iron that can be reused and stored for ferritin complexes. Remove any bacteria or debris that is present in the portal blood.	(Junqueira 2003)
Hepatic Stellate Cells	Small lipid droplets that store fat soluble vitamins and vitamin A. Help regulate Kupffer cell activity.	(Junqueira 2003)
Hepatic Portal Vein	A branch of the portal vein with blood that's rich in nutrients but low in O_2.	(Junqueira 2003)
Hepatic Artery	A branch of the hepatic artery that supplies O_2.	(Junqueira 2003).
Bile Ducts	Facilitates in bile flow from the bile canaliculi to hepatic ducts.	(Junqueira 2003).

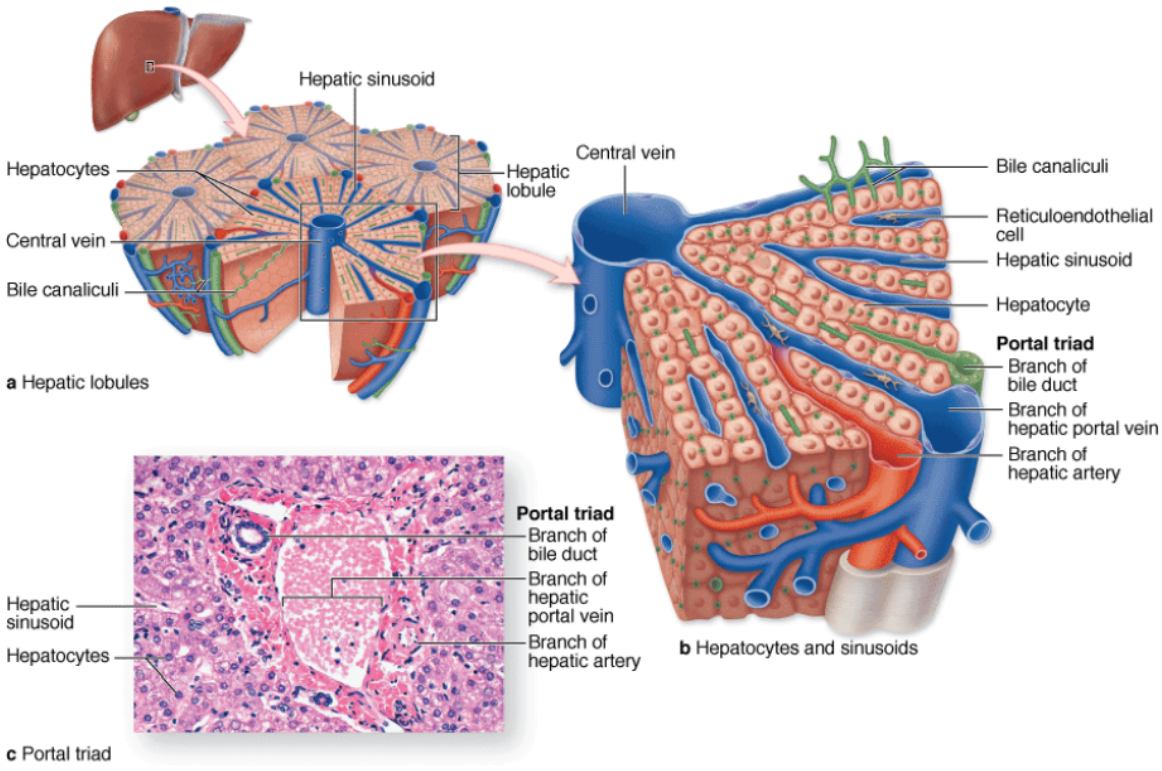


Figure 2: Models of the liver microstructure. (A) The liver is composed of thousands of polygonal structures that create hepatic lobules. (B) Three-Dimensional diagram of hepatocytes, sinusoids, central vein, and the portal triad. (C) Micrograph of hepatic lobule containing the central components of liver structure (Junqueira 2003).

Model Organisms

Goldfish (*Carassius auratus*) are a common model organism used for the regulation of reproduction in vertebrates and neuroendocrine signaling as well as in various other fields (Popesku 2008). They are teleost fish that are a well domesticated (Tsai 2013). Goldfish are a part of the minnow and carp family which is the largest family of fishes (Moyle 2000). They typically have a body plan that has moderately deep bodies, conspicuous scales, large eyes, abdominal pelvic fins, and small terminal mouths (Moyle 2000). Because of the historical background and morphological features, goldfish are a commonly used model organism (Tsai 2013).

The liver of fish, which is typically a reddish brown or yellow color, differs from mammalian liver because hepatocytes do not typically form cords, lobules, or portal triads that are obvious (Mumford 2007). A histological example of a typical fish liver is presented in Figure 3. The liver of Goldfish is mainly composed of hepatic plates which are basically two layers of hepatocytes (Nopanitaya 1979). Mammalian hepatocytes differ from the goldfish hepatic plates but are morphologically similar to goldfish parenchymal cells (Nopanitaya 1979). The liver of many fish is one of their most damaged organs (Mumford 2007).

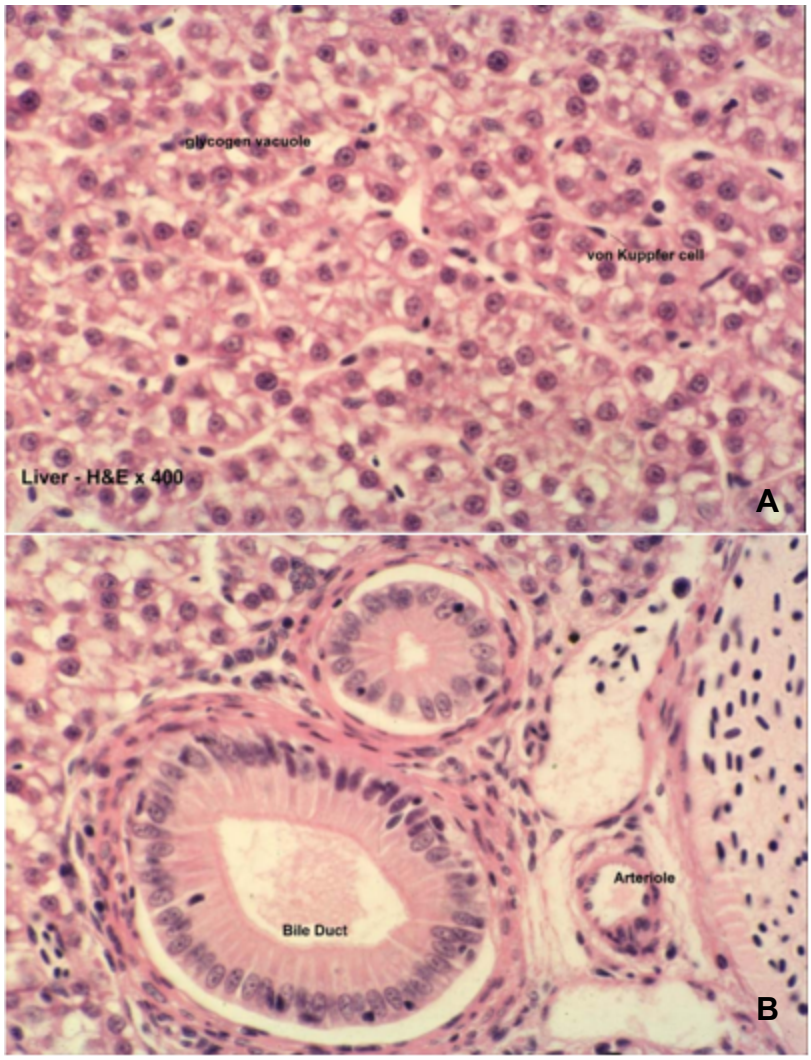


Figure 3: Example of a normal liver of Goldfish (Carassius auratus). (**A**) emphasizes a glycogen vascuole and a Van Kuppfer Cell. (**B**) has the Bile duct and arteriole marked on the image (Mumford 2007)

Another common model organism being used in this study is the African Clawed frog tadpoles (*Xenopus laevis*). This smooth skinned, toad-like creature was first discovered by Daudin in 1803 (Deuchar 1991). Unlike most other frogs or toads, African Clawed frogs spend the entirety of their life as aquatic animals (Deuchar 1991). *Xenopus laevis* tadpole's herbivorous diet mainly consists of algae, while their adult counterparts' diet consists of crustaceans, worms, and other small creatures that can be found on the mud (Wilt 2017). This species is able to be managed in a laboratory setting easily, breed quickly which result in

many offspring and are inexpensive (Wilt 2017). Another characteristic that makes them a model organism is that they can survive in captivity for a long time and have relatively low mortality rates (Wilt 2017). Because of their rapid reproduction and maturation rates, they are commonly used in research (Deuchar 1991).

More specifically, *Xenopus laevis* is used mainly in developmental biology studies (Wilt 2017). In a study by Sadaghiani, the neural crest development of an African Clawed frog embryo was observed by scanning electron microscopy (1987). They also were used to study the development of erythroid progenitors that respond to erythropoietin in the larval livers of *Xenopus laevis* (Okui 2016). African Clawed frogs also have more extensive liver compared to other species of frogs (Deuchar 1991).

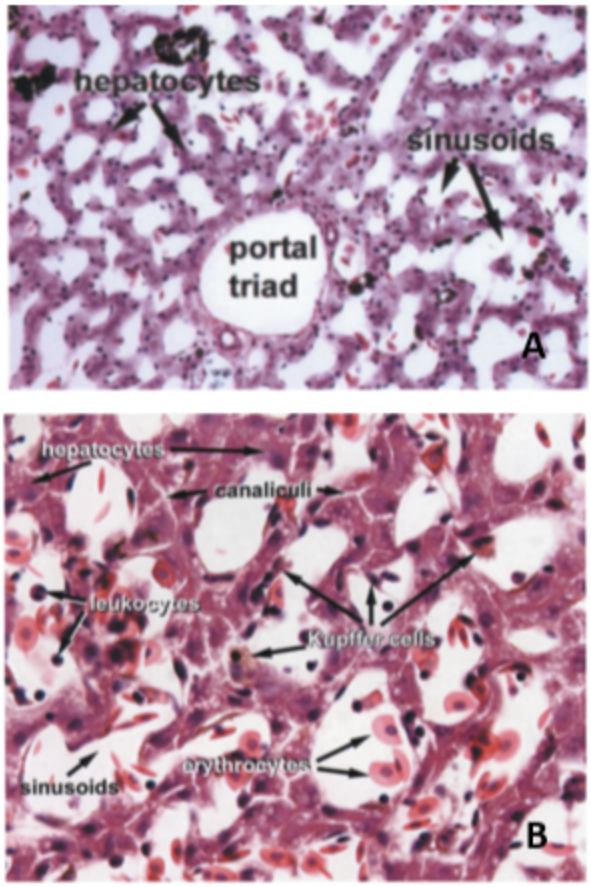


Figure 4: Example of a normal liver of African Clawed Frog (*Xenopus laevis*). (A) Major cellular components of *Xenopus laevis* liver including the hepatocytes, sinusoids, and portal triad. (B) Magnified view of the more specific cellular components of the *Xenopus laevis* liver. (Wiechmann 2003)

Purpose

The aim of this study is to identify the effects of EGCG, an active ingredient in green tea extract, on liver structure in Goldfish (*Carassius auratus*) and African Clawed Frogs (*Xenopus laevis)*. The liver structures of the exposed Goldfish and African Clawed frogs will be compared to the livers of unaffected animals of the same species to identify the changes that occurred due to EGCG exposure.

CHAPTER II

MATERIALS & METHODS

Study Animals

Animal Husbandry

Goldfish (*Carassius auratus*, n=10) were purchased from Pet Supplies Plus in Maryville, TN and housed in 500ml of dechlorinated water that was treated with Jungle Start Right® complete water conditioner. Stage 56-63 African Clawed frog (*Xenopus laevis,* n=10*)* tadpoles were purchased from NASCO and housed individually in 500ml beakers. Goldfish were fed 1 TetraFin Goldfish Flake and 1 Wardley Goldfish Pellet and the African clawed tadpoles were fed NASCO Frog Brittle.

Exposure

During this experiment, five goldfish and five African clawed frogs where exposed to 100µg/L (-) epigallocatechin-3-gallate (EGCG) for 14 days. First an ECGC stock (Sigma Aldrich; CAS #: 989-51-5) was created by adding 100mg of EGCG to 50ml of water (2 mg/ml stock). In order to ensure that the solution was mixed thoroughly, a stir bar was placed in the beaker and stirred for a minute. In order to get a final EGCG concentration of 100µg/L, 25µL of 2 mg/ml EGCG was added into the 500ml that each goldfish and tadpole where housed in. Each fish or tadpole was placed into its own labeled container exposed to EGCG once a day.

Euthanasia

After 14 days, tadpoles and Goldfish were euthanized using MS-222 at concentration 500mg/L. Livers were removed using microdissection and fixed in Bouin's fixative.

Histology

The technique of histology was then used to analyze the livers of both species. This process consisted of six steps including fixing, embedding, sectioning, staining, mounting, and analyzing the organ sample.

Embedding the Organ

After clearing the excess fixative from the sample, the liver was dehydrated by transferring the sample to increasing concentrations of alcohol, followed by a clearing agent. The process of transferring the tissue into various concentrations of alcohol and wax for 1 hour increments was performed as outlined below. Once removed from Wax IV, the tissue was placed into a warmed wax pot and embedded (insert the direction that I embed them) in a paraffin wax block to prepare for sectioning.

80%.....1 hour
95%.....1 hour
100%.....1 hour
100%.....1 hour
SafeClear.....1 hour
SafeClear 2.....1 hour

Wax I.....1 hour @ 12in. Hg
Wax II.....1 hour @ 12in. Hg
Wax III.....1 hour @ 12in. Hg
Wax IV.....1 hour @ 12in. Hg

Sectioning and Mounting

The paraffin wax block containing the liver was trimmed down around the organ into a pyramidal shape. The block was mounted onto the microtome and sectioned into 12 μm-

thick ribbons. The ribbons produced from the tissue were placed onto a paper towel in the order of which they were cut. Next, pieces of each ribbon were placed into a warm water bath containing a pinch of gelatin. The ribbons were then mounted on the slides, labeled, and allowed to dry on a slide warmer.

Staining

The stains that are used to visualize the various cell structures are Hematoxylin and Eosin. Once the slides are dry, the H&E procedure as represented in Figure 5. was performed. Once the staining procedure was complete, coverslips were placed on the slides with Permount.

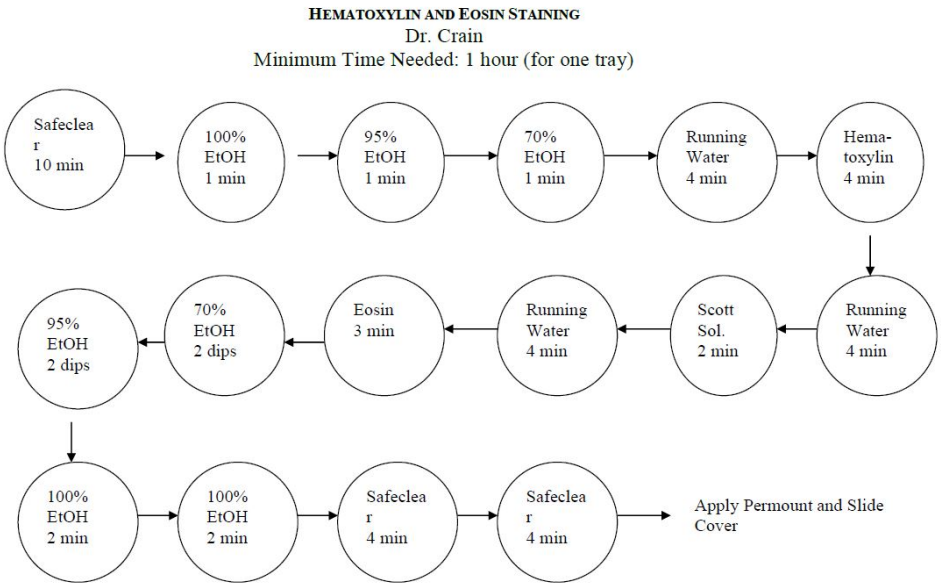


Figure 5: Hematoxylin and Eosin staining procedure. (Crain 2019).

Outcomes Measured

Behavior

All fish and tadpoles were observed during the 14-day exposure period. Their behavior was recorded and denoted the swimming actions of each organism. This includes

whether or not the organism is swimming right side up, upside down, swimming slower or faster, and of it is lethargic in any way. Any other unusual actions were also recorded. All behaviors where then compared to a behavior scale.

Liver Histology

The slides were allowed to dry overnight prior to analysis. Orientations of the organs were noted, and digital photographs were taken of the liver sections in a light microscope with an attached Nikon camera. Measurements of 5 hepatocytes from a control goldfish and a goldfish exposed to EGCG were taken. Measurements of 5 hepatocytes from 3 different control tadpoles and tadpoles exposed to EGCG were taken.

Statistical Analysis

Excel was used to analyze all data. Multiple t-tests assuming equal variances where run on all weight data. The measurements of the hepatocytes for the goldfish were averaged; however, a t-test could not be ran due to a small sample size. Measurements of the hepatocytes for the tadpoles were average and then a t-test assuming equal variances was ran to analyze the data.

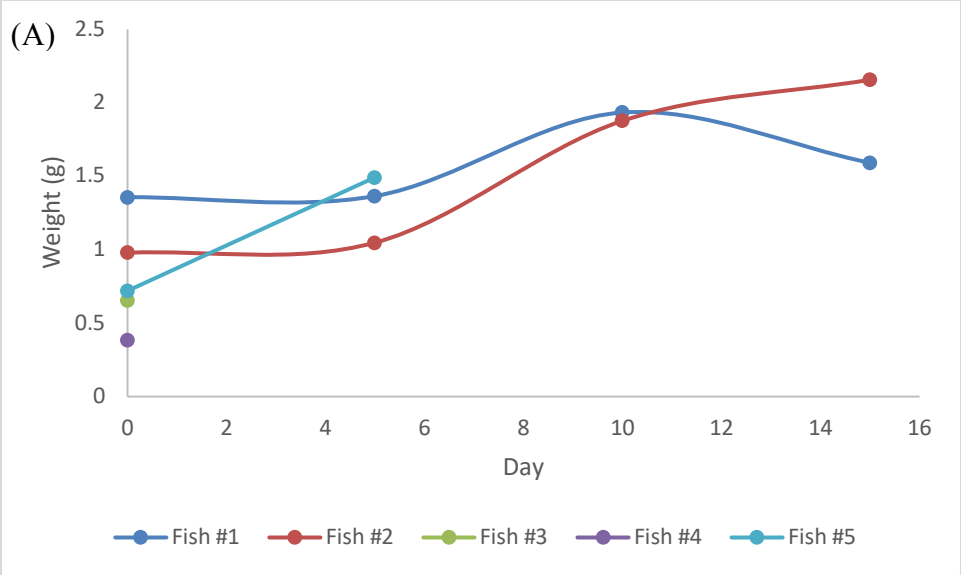
CHAPTER III

RESULTS

Both qualitative and quantitative analyses were conducted on liver sections of goldfish (*Carassius auratus*) and tadpoles (*Xenopus laevis*).

Weights

The weights of the control and (-) epigallocatechin-3-gallate (EGCG) exposed goldfish and tadpoles are shown in Figure 6 and 7, respectively. The weight for the goldfish showed no significant difference between control or treated individuals (see Table 4), whereas tadpole weight from day 0-10 is near significance($p=0.07$) with treated individuals losing more weight (Table 4).



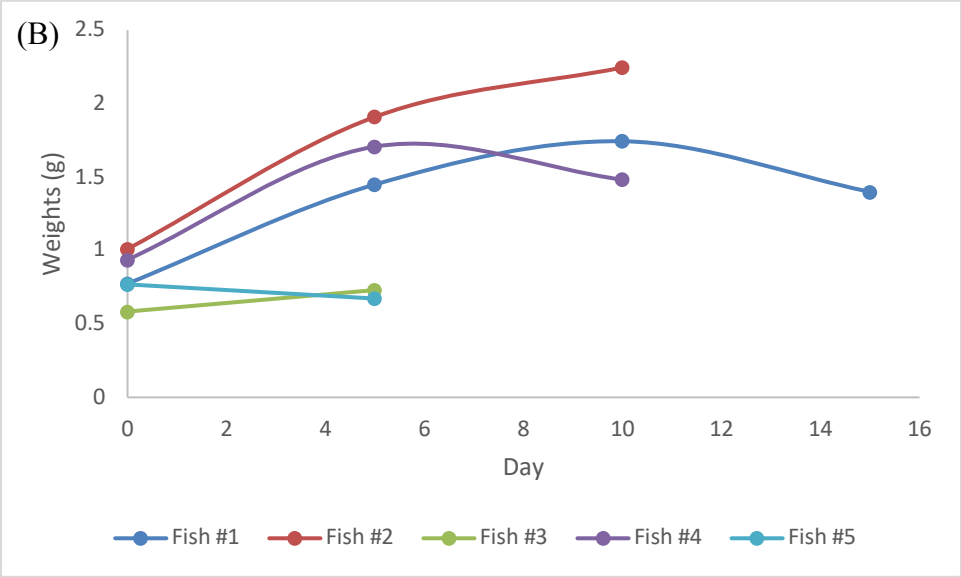


Figure 6: (A) The progression of weights for the control Goldfish (*Carassius auratus*) 1-5 on days 0,5,10, and 15 of the experiment. (B) The progression of weights for the Goldfish (*Carassius auratus*) 1-5 that were exposed to (-) epigallocatechin-3-gallate (EGCG) on days 0,5,10, and 15 of the experiment.

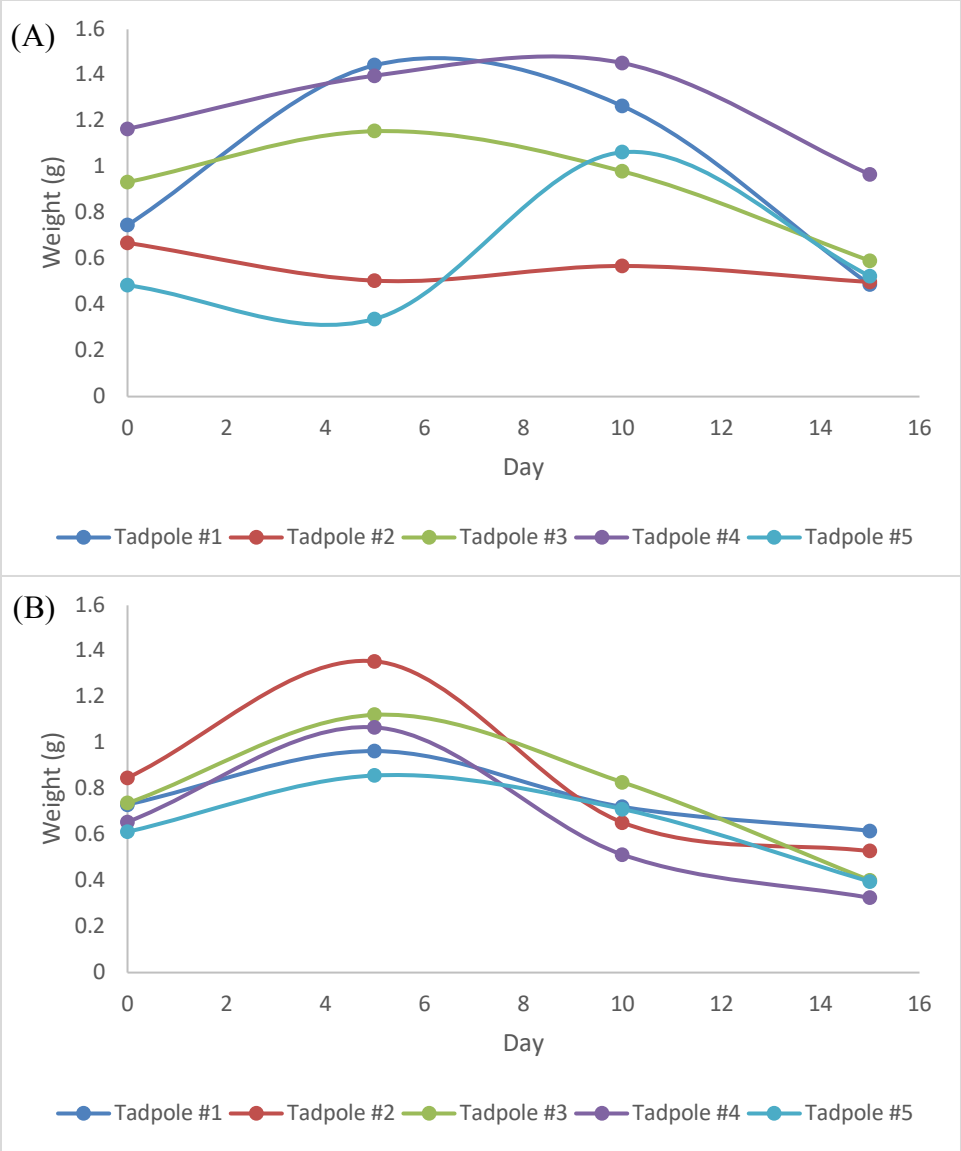


Figure 7: (A) The progression of weights for the control Tadpoles (*Xenopus laevis*) 1-5 on days 0,5,10, and 15 of the experiment. (B) The progression of weights for the Tadpoles (*Xenopus laevis*) 1-5 that were exposed to (-) epigallocatechin-3-gallate (EGCG) on days 0,5,10, and 15 of the experiment.

Table 4: The significance values of the compared weights on days 0-5, 0-10, and 0-15 for the goldfish and tadpoles.

Day	Goldfish	Tadpoles
0 to 5	0.13	0.288588
0 to 10	0.540389	0.072952
0 to 15	0.344765	0.345267

Behavior

The behavior of all of the tadpoles was very consistent throughout the entire experimental period. They would swim normally or would lie at the bottom of their beaker very still and only move when the beaker was moved. This was consistent for both control and EGCG exposed tadpoles. However, for the goldfish activity was not the same. Only control goldfish 1 & 2 and EGCG exposed goldfish 1 remained normal throughout the exposure period. For all of the other goldfish, both control and exposed, there was no food consumed within the hours before dying. Neither the flake nor pellet they had been given where eaten every time. Another side effect that happened to only the EGCG exposed goldfish where that before dying, whether it was within a few hours to death or a few days, the goldfish would start swimming on its side. It seems like they were having a hard time breathing and staying afloat because they would sink to the bottom and try to swim towards the top of the beaker. This process was repeated until the would die. So, while the behavior of the tadpoles was not influenced by EGCG, there is some evidence that the behavior of goldfish may have been affected.

Histological Data

Measurements were taken of the hepatocytes for the different organisms. The hepatocytes for the control goldfish (see Figure 8) had an average size of 7.7μm, whereas the average hepatocyte size of the EGCG exposed goldfish (see Figure 9) was 5.75. This difference appears to be significant (see Figure 10), but due to a sample size of only one for each group, a statistical test could not be conducted. For (*Xenopus laevis)* tadpoles, there was no difference ($p=0.74$, see Figures 11-13).

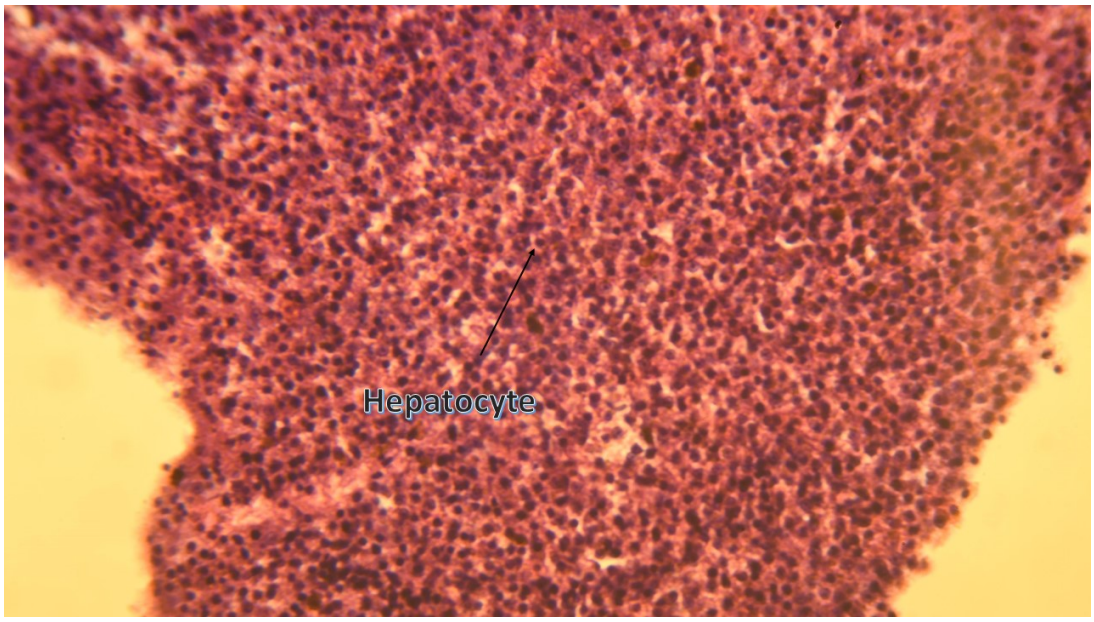


Figure 8: Liver section of a control (*Carassius auratus*) goldfish showing a hepatocyte at 400x magnification.

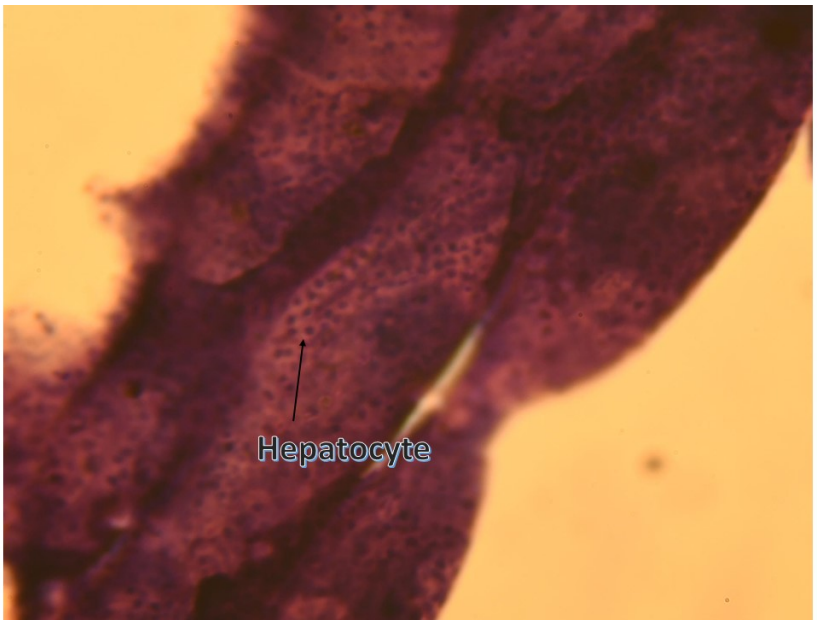


Figure 9: Liver section of an EGCG exposed (*Carassius auratus*) goldfish showing a hepatocyte at 400x magnification.

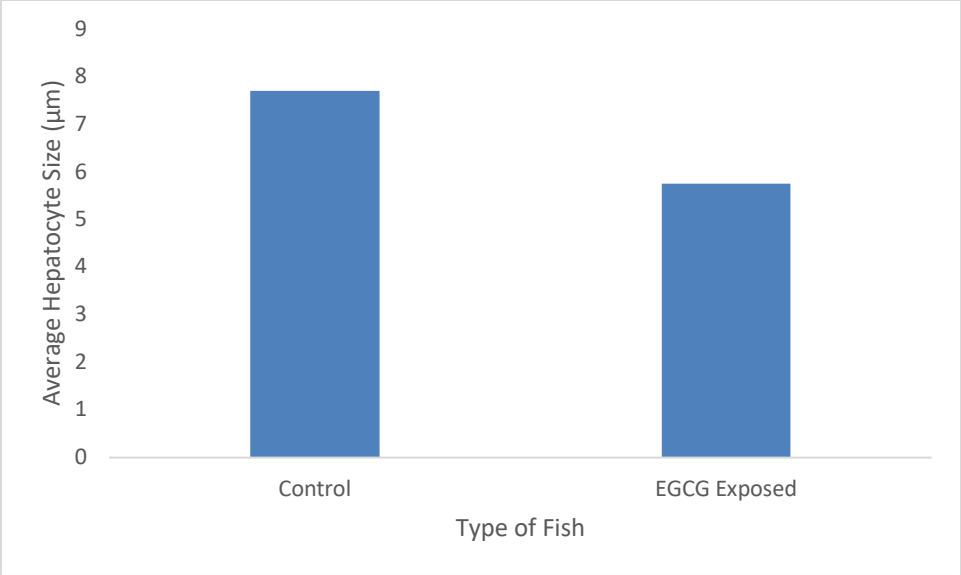


Figure 10: The average size of five counted hepatocytes size from control and EGCG exposed goldfish.

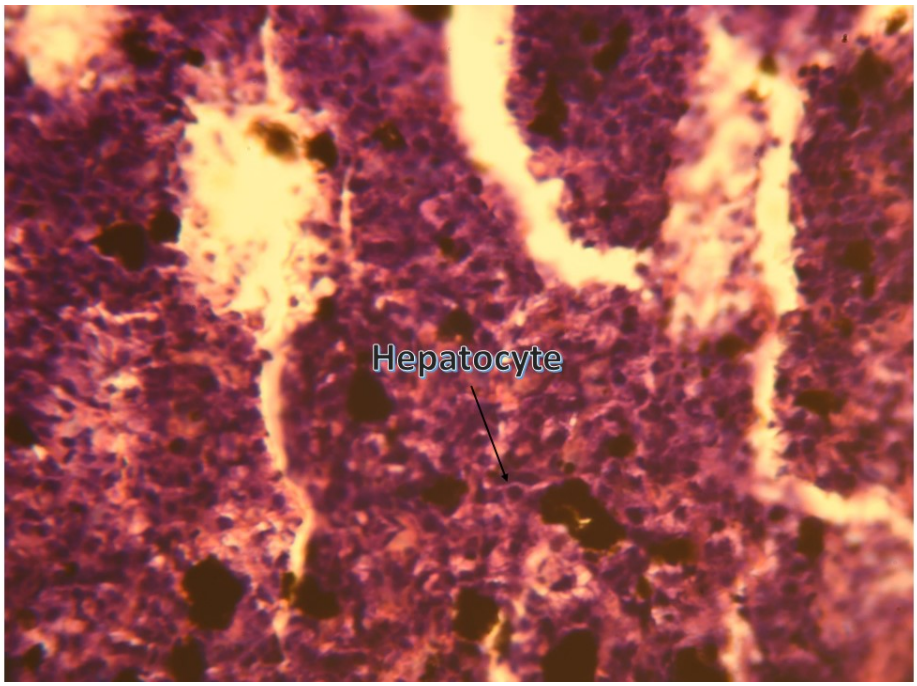


Figure 11: Liver section of a control (*Xenopus laevis)* tadpole showing a hepatocyte at 400x magnification.

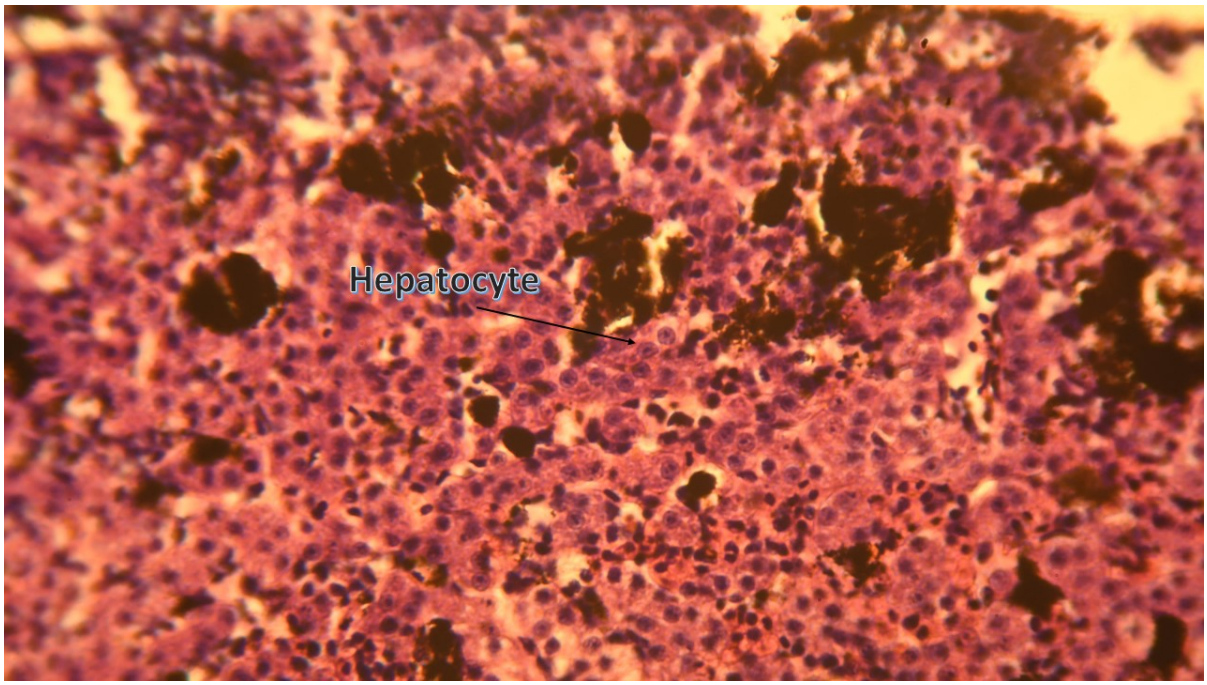


Figure 12: Liver section of an EGCG exposed (*Xenopus laevis)* tadpole showing a hepatocyte at 400x magnification.

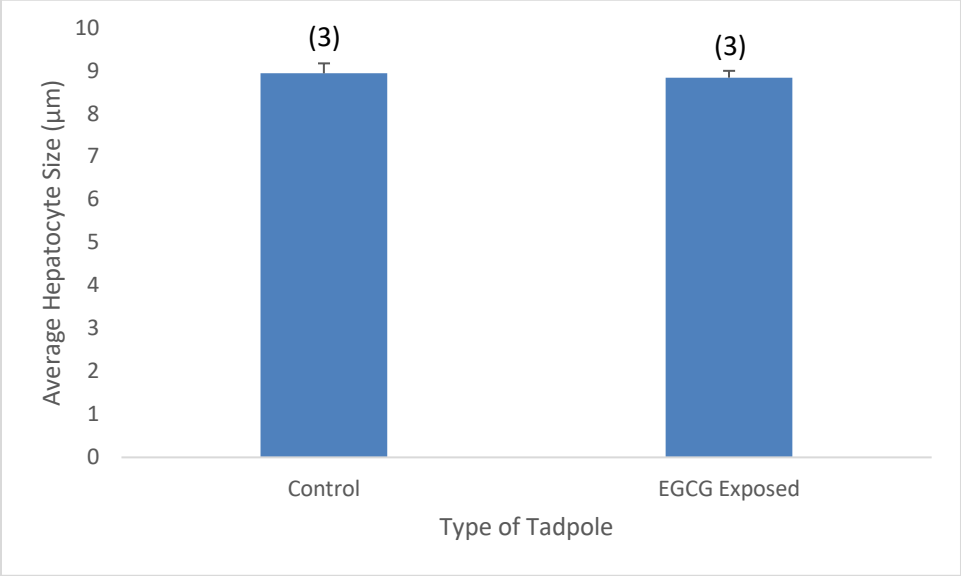


Figure 13: The average hepatocyte size of control (*Xenopus laevis)* tadpoles (8.966µm±0.2333SE) and EGCG exposed tadpoles (8.866µm ±0.158SE). P=0.74.

CHAPTER IV

DISCUSSION

The weights of all the goldfish and many of the control tadpoles fluctuated throughout the experiment; however, many of the EGCG exposed tadpoles lost weight throughout the 15-day process. Weight loss in metamorphosing tadpoles is normal, as the budding of the forelegs and hindlegs during does not occur until weight loss has occurred (Adolph 1931). This critical weight loss is a precursor for the frogs to go on to the next stage of metamorphosis. Similar weight loss mechanisms are seen in other species that go through metamorphosis, like in the solitary bee pollinators, *Osmia lignaria* (Helm 2017). Attaining the critical weight again cues metamorphosis and hormone signaling (Helm 2017). Because green tea extract is typically used as a weight loss supplement, exposure was expected to cause weight loss.

In mammals, the weight loss is most likely due to green tea extract inducing thermogenesis (Shixian 2006). In male Wistar rats exposed to green tea extract for 5 days a week for 12 weeks, obese rats showed a significant reduction in fat synthesis and body weight as well as hyperlipidemia and fat depots (Rocha 2015). In these mammals, green tea extract activated the AMPK, which likely reduced the concentrations of plasma and liver lipid content, stimulating fatty acid oxidation in the same tissue (Rocha 2015). Therefore, it

is likely that this is the mechanism that helps cause weight loss in humans who take EGCG supplements.

This pathway and reasoning for how EGCG causes weight loss in mammals could not be the same for tadpoles who, as ectotherms, do not have the ability for endogenous thermogenesis. Thermoregulation is only possible behaviorally in ectotherms. Their thermoregulatory sensors receive signals that stimulate transcriptional regulators of metabolic processes (Flouris 2014). The growth rates and ages at metamorphosis as well as sexual maturation and death all depend on metabolic rate (Flouris 2014). Thus, the weight loss in these organisms in this experiment could have purely been due to metamorphosis. Another possible mechanism of how EGCG could have induced weight loss could be due to its neuroprotective and stimulatory affects (Singh 2016). In humans, EGCG has been known to cause and increase in neuronal stimulation, so this neuronal stimulation could have also occurred in the tadpole causing them to send signals to the thermosensory sensors and stimulate metabolism (Singh 2016). This may show that animals that can't have induced thermogenesis like in mammals may not be affected by EGCG in the same capacity or intensity. This could also explain why there were no effects seen in the size of hepatocytes for the control and treated tadpoles and fish.

In a study done by Mezra et al. (2014), male Wistar rats were subjected to a partial hepatectomy or sham operation and their hepatocytes where then isolated treated with various concentrations of EGCG. They found that concentrations of 10µmol/L were toxic to hepatocytes regardless of the partial hepatectomy or sham operation (Mezra 2014). Another study found that when rats were exposed to EGCG when the concentration of the dosage was lower than 10µmol/L, the hepatocytes where normal (Kucera 2015). However, at a dosage of

10μmol/L or more there were more signs of cellular injury as well as a decreased function in hepatocytes (Kucera 2015). EGCG was also a factor in increased apoptosis in the liver cells of the rats , as it induced the activation of the intrinsic pathway of apoptosis (Kucera 2015). The leading mechanism for such hepatocyte mitochondrial damage is due to its production of a large number of free radicals (Sánchez 2012). Also, hepatoxicity effects will be increased if someone is consuming large amounts of green tea as well as alcohol (Sánchez 2012). This is due to green tea increasing the average life of ethanol levels in the blood and therefore creating higher toxicity levels (Sánchez 2012).

Whereas excessive exposure to EGCG causes the aforementioned harmful effects, there are many beneficial effects of EGCG. One study showed that EGCG may be useful in the treatment and prevention of hepatic fibrosis in rats by reducing the activities of serum alanine aminotransferase and aspartate aminotransferase (Zhen 2007). Another study aimed to examine the effects of EGCG on changes in body composition, energy and substrate metabolism and liver function enzymes after an energy restricted diet intervention in obese women (Mielgo 2013). This study was a double blind, placebo controlled, randomized study that used 83 obese women and determined that supplementation of 300mg/d of EGCG for 12 weeks did not cause any harmful effects on liver function biomarkers (Mielgo 2013). Thus, much more research must be done on the effects of EGCG to determine if the benefits outweigh the risks.

Due to studies like the present one, more political and health professionals are paying closer attention to the advertisements and promotions of “natural supplements” like EGCG. Health Canada has begun ordering more explicit warning labels for green tea extract products due the risk of liver toxicity (Government of Canada 2017). This was instigated due to the

increasing reports of liver injury caused by green tea extract in Canada and worldwide (Government of Canada 2017). Because supplements like EGCG claim to help people lose weight, having greater restrictions and paying closer attention to its dosage and distribution is important in regard to someone's livelihood. This is also why in 1994 in the United States FDA began regulating dietary supplement products and dietary ingredients (Center for Food Safety and Applied Nutrition 2019). These regulations included prohibiting products that are adulterated or misbranded and being responsible for evaluating the safety and labeling of those products (Center for Food Safety and Applied Nutrition 2019). This lead to countries like France and Spain prohibiting the commercial distribution of green tea based products in 2003, the United States Pharmacopeia systematically reviewing the safety of green tea consumption in 2008, and the constant increase in public health measures to combat the rising numbers of people being affected by liver toxicity and damage due to polyphenols in green tea extract (Sánchez 2012).

Future studies should explore mammalian models of human exposure, as this study realized that ectothermic organisms are not appropriate due to the mechanisms of action of EGCG and explore higher dosages. Such studies would allow for a better understanding of the pathways of how EGCG effects the liver and other aspects of weight loss and metabolism. Because many studies have claimed that only after a certain dosage of EGCG can you see effects on the liver, a more advanced way of doing this would be to have three or four different groups, with each group being exposed to a different concentration/dose of the EGCG. This would give more insight to what specific dosing causes liver damage because as of right now, it seems like 10µmol/L is the turning point of when EGCG becomes toxic. In the future this experiment could be changed in order to see the effects of EGCG on other

organs as well as testing to see if other components of green tea or a different type of tea would have on the liver.

The health and dietary supplement market is so expansive that being able to fully understand what goes into these products is pertinent. Knowing exactly how (-) epigallocatechin-3-gallate (EGCG) effects the liver and other organs could help prevent more people from hepatoxicity and overall increase the health literacy of people in regard to what they're putting into their bodies. Studies on EGCG in mammalian model species are warranted.

APPENDIX 1: IACUC Approval Form

MARYVILLE COLLEGE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE (IACUC)
Application for Use of Vertebrate Animals in Faculty Research or Teaching

Faculty at Maryville College that use vertebrate animals in teaching or research are required to complete an IACUC proposal for each project.

Provide information after each bold item
Faculty Name: Dr. Drew Crain

Student Name: Miracle Walls

Email Address: miracleann.walls@my.maryvillecollege.edu

Date: March 11, 2019

Species to be used: Zebrafish *(Danio rerio)* & African Clawed Frog/tadpoles *(Xenopus laevis)*

Age of animals: Adult zebrafish and Tadpoles will be staged using Gosner Staging.

Number of animals in study: 10 Zebrafish and 10 Tadpoles will be used.

Brief description of use (teaching or research):

Duration of use: The animals will be exposed to the experimental conditions for 15 days.

Location of animals (building and room): Sutton 114

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Miracle Walls	(901)-614-7628	(901)-614-7628	(901)-614-7628
Dr. Drew Crain	(865) 981-8238	(865) 981-8238	(865) 981-8238

What will happen to the animals at the end of the use? If euthanasia is required, state the methods.

After being exposed to a dilution of green tea extract for 15 days, the zebrafish and the tadpoles will be anesthetized with MS-222 for the removal of their liver. I will have to physically

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

Maryville College IACUC Approval Number: _201905_____
Date Approved: __3/31/19_____
Signed: _____

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