THE EFFECTS OF CANNABIDIOL (CBD) ON EPITHELIAL DEVELOPMENT OF THE WOOD FROG (*LITHOBATES SYLVATICA*)

A Report of a Senior Study

by

Kelsey Birchfield

Major: Biology B.S.

Minor: Chemistry

Maryville College

Fall, 2021

Date approved Nov. 19, 2020, by _____

Faculty Supervisor

Date approved Nov. 19, 2020, by _____

Division Chair

ABSTRACT

Recently, cannabidiol, commonly known as CBD, has become a growing industry that provides hope for inflammatory diseases and illnesses. However, the FDA does not regulate CBD and little research has been conducted to determine its effects. This study utilized Wood Frogs (*Lithobates sylvaticus*) tadpoles and newly metamorphosed frogs to determine developmental effects CBD may have on the epithelial system. Half of each group were fed CBD-infused Navisco Frog Brittle for 16 days were anesthetized and fixed on the last day. A few weeks after fixation, the metamorphosed frogs and tadpoles were embedded, cut to slides, and stained for analysis. Epithelial density, outer cell height and width, squamous cell height and width, mucosal height and width, mucosal gland diameter, and epithelial thickness were all quantified. Statistical analyses found that exposed tadpoles had a lower density ($p<0.001$), a shorter squamous cell height ($p<0.001$), and shorter and wider mucosal cells ($p<0.001$) than control tadpoles. Exposed frogs had shorter and wider outer cells than the controls ($p<0.001$). Observationally, exposed frogs and tadpoles seemed to be less reactive than controls and 2 exposed tadpoles had severe spinal deformities while another exposed tadpole was 15 stages behind the rest. These results suggest that CBD does affect epithelial development and should be researched further to evaluate its possible impact on humans and other animals.

ACKNOWLEDGEMENTS

I would first like to acknowledge my advisor, Dr. Drew Crain, for leading me in my research journey while providing constructive criticism and words of encouragement throughout the writing process. I would also like to thank Dr. Duncan for guiding me through the chemical methods used. Lastly, I would like to appreciate Bryan Crouser, Julia Warrick, and Miracle Walls for their support, advice, and helpfulness in the analysis and histological fixation process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The Benefits of Cannabidiol

During the last 20 years, cannabidiol, a prevalent compound in *Cannabis sativa*, has been recognized as a therapeutic for several medical purposes. Commonly referred to as CBD, the extract has been used to evaluate potential antipsychotic, epileptic, and proapoptotic benefits in various studies (Bergamaschi et al., 2011). Additionally, cannabidiol has become a major focal point on the recent research conducted on inflammation and chronic inflammatory diseases. Many of these studies have used animal models such as transgenic mice to demonstrate the potential benefits of CBD *in vivo*. Human studies are reserved or forbidden due to laws in the U.S. discussed later (Mead 2017). A list of compiled medical benefits of CBD from previous studies can be found in Table 1.

Table 1. Benefits of cannabidiol regarding medical diagnoses.

Medical Diagnosis	Benefit	Reference
Myocarditis	Significant decrease in T cell count, inflammation, myocardial fibrosis, and cardiac dysfunction	(Lee et al., 2016)
Multiple Sclerosis	Slows neurodegeneration and progressive disability	(Pryce et al., 2014)
Cocaine Addiction	Reduced paranoia, cravings, withdrawal, and lack of impulsiveness	(Fischer et al., 2015)
Epilepsy	Reduction of motor seizures	(Devinsky et al., 2015)
Anxiety	Decrease in generalized anxiety and invasive thoughts	(Shannon et al., 2019)
Schizophrenia	Decrease in psychosis and anandamine blood levels	(Batalla et al., 2019)
Cannabis addiction	Reduced cravings and withdrawals, lower overall use	(Batalla et al., 2019)
Alzheimer's Disease	Prevents neurodegeneration of the hippocampus and cortex	(Watt and Karl 2017)

Although all CBD analogs are derived from the same bent backbone of 2 phenyl groups, the identified side groups consisting of n-pentyl, n-butyl, n-propyl, and methyl provide differing affinities for receptors of interest, allowing CBD to be easily manipulated and used in the medical settings. The possibilities of these side chains are shown in Figure 1. Because of its bent conformation, shown in Figure 2, CBD cannot bind and activate CB1 receptors like its plant counterpart, Δ^9-tetrahydrocannabinol (THC) can, because there is too much steric hindrance. This accounts for the lack of psychotic effects and short-term memory loss experienced by THC (Burstein 2015).

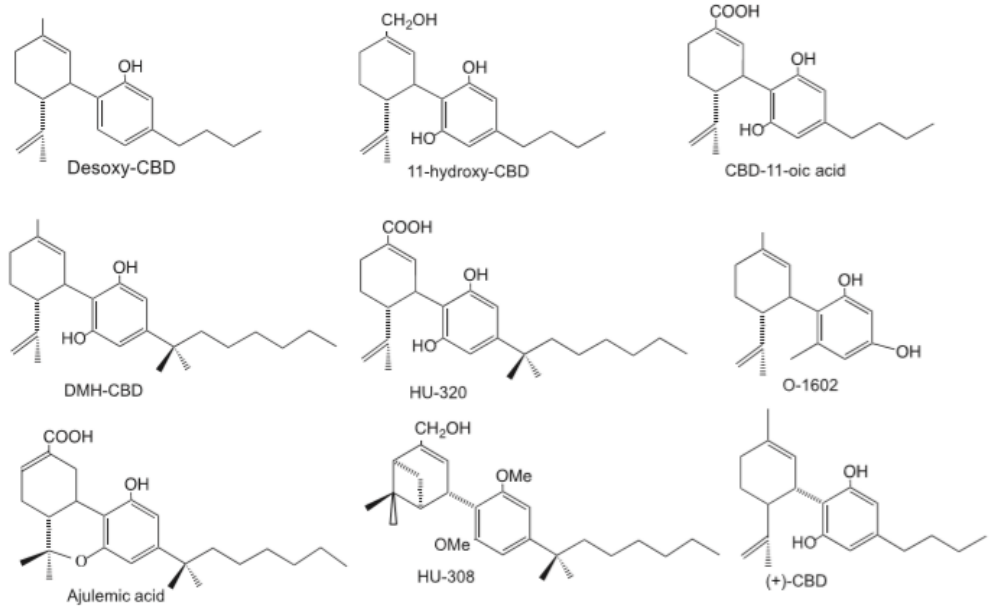

Figure 1. Various side chains and structural arrangements of cannabidiol found *in vivo* (Bernstein 2015).

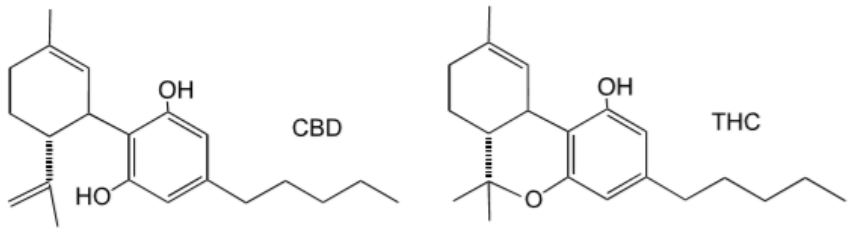

Figure 2. Structural differences of cannabidiol and Δ^9-tetrahydrocannabinol. The bentness of CBD creates steric hindrance, making it unable to bind the CB1 receptor (Bernstein 2015).

The Legality and Regulation of Cannabidiol in the U.S.

Although CBD has been heavily researched recently, the laws regarding medical use of the compound in the U.S. are tricky and sometimes forbid use. Because cannabidiol is a cannabinoid found in *C. sativa*, it is hard to isolate CBD from THC, which causes a legal issue that can be on the federal or state level. Some states allow THC to be sold for medical purpose while others only allow *C. sativa* substances to be sold if it is mostly cannabidiol with a very low THC concentration. Although medical THC is legalized, it is considered a Schedule I drug under the Controlled Substance Act (CSA), grouped under a list of other drugs with abuse potential such as heroin and LSD (Mead 2017). This makes cannabinoids unavailable via prescription, but available for use during research studies. Because of this, physicians may only suggest or recommend to a patient that CBD may be a helpful treatment for a condition. Prescribing patients CBD or THC is considered illegal on the federal level (Mead 2017).

Even these research studies have strict rules on CBD:THC ratios and those who grow must be under contract with the government. However, because these ratios are not regulated medically because the FDA has not approved and does not regulate the sale and use of cannabinoids, medical cannabinoid concentration varies, and preparation and growth is

uncertain. This creates a problem for users and the potential for incorrect dosage. Additional issues arise when state lines are crossed carrying what was legal for one state, now illegal. This is not only a complication families and patients face, but also nurses and caregivers (Mead 2017).

This lack of FDA regulation has led to warnings being issued to vendors and buyers because the CBD content is lower or higher than advertised. Additionally, because CBD is lumped in with the THC definition of a Schedule I drug despite its non-psychotic properties, lack of presence in locations *C. sativa* is defined, and industrial hemp being farmed by states, it cannot be overtly marketed as a supplement because it is against federal law (Corroon and Knight 2018, Mead 2017).

The Development of *Lithobates sylvatica*

Lithobates sylvatica, the wood frog, is a 4-8 cm frog that can be found in North American locations from Western Alaskan ponds to the Appalachian mountains in Eastern Tennessee (Meeks and Nagel, 1973). In east Tennessee, wood frogs lay 2,000-4,000 eggs that are fertilized externally by males. The fertilized eggs are deposited approximately 33 cm below the surface of water, with egg masses between 28-100 mm in diameter. These embryos typically will hatch after incubating for 18-20 days and then grow for another 84 days until they metamorphose into an adult frog (Dodd Jr. 2004; Meeks and Nagel, 1973). Size and phenotype of the tadpoles are dependent on temperature; exposure to higher temperatures during the egg and larval periods results in tadpoles that are significantly smaller, have shorter tails, and swim slower than their counterparts exposed to lower temperatures (Watkins and Vraspir 2005). Higher temperatures promote quicker swimming and a larger body size, but tadpoles incubated in higher temperatures metamorphize quicker, making the

chance of survival higher (Watkins and Vraspir 2005). Slower developmental rates have also been recorded when the tadpoles are in the presence of higher densities of *Phragmites australis*, while faster developmental rates have been noted with higher densities of *Phalaris arundinacea* (Perez et al., 2013). Interestingly, tadpoles that have been hatched with predators present develop slower, are significantly smaller, and also have deeper and longer tails than those raised with no predator threat (Relyea 2002).

Tadpoles are dark gray or brown with a faint white stripe on the upper jaw, making *L. sylvatica* easy to identify because they are the only dark tadpoles present during spring and initial summer months. As these tadpoles grow, they mature to be a light to dark brown color with a black mask reaching from the eyes to the forelimbs. The white stripe of the tadpole's upper jaw is maintained throughout maturity and the stomach whitens. Dark brown bars may be present or absent on the outer rear legs (Dodd Jr. 2004).

The sex of the frogs is ultimately determined genetically, yet environmental factors during the larval and embryonic stages reign over sexual differentiation. At 20°C, there is a 1:1 ratio of male to female sex outcome. Embryos at 32°C develop as solely male and this temperature causes no effect on males in the late larval stage, yet female reproductive structures stop developing and are replaced by the growth of seminiferous tubules. Because of the late development of the male structures, the frogs are intersex and have seminiferous tubules as well as non-functioning oocytes from the degenerative oviduct (Lambert et al., 2018).

Wood frogs breed once fully mature in the early spring/late winter. Because wood frog egg masses are deposited in water on cold nights, the frogs must migrate from their leaf litter or woodlands to breeding sites. Males migrate to local ponds or pools in the late fall to

secure their chance at fertilizing females, while females migrate to the ponds only when it is nearer to the breeding season (Regosin et al., 2003). Although their breeding season is very short-lived (only a few days of the year), it is explosive. This explosion is characterized by wood frogs scrambling to find breeding ponds of their species based upon choral noise. Males use the conspecific chorus of their species to locate these ponds for annual breeding and tend to avoid heterospecific choruses so they have better odds at reproduction (Bee 2007). Because there is so much male competition for female breeding in such a short period, there have been many studies reporting dead adult *L. sylvaticus* in or near breeding sites. These happenings occur due to "mating balls" formed by several male frogs mounting a female at one time and smothering her (Trauth et al., 2000). Sometimes the female will survive shortly after the smothering but will be unable to reproduce due to organ damage. These frogs typically die via predation or are overtaken by parasites. Similarly, weaker males have also been found dead, presumably due to their loss in these mating balls.

The Skin of *L.sylvatica*

Frog skin is a topic well-researched that evaluates the incredible efficiency and diversity frogs have. The skin is composed of an epidermis, a thin layer of stratified squamous epithelia covering the outside of the body, and a dermis, a skin component directly under the epithelium, anchoring these layers to the inner structures of the frog via columns of collagen. The epidermis is composed of the stratum corneum, central stratum spinosum, and the stratum germinativum in order of descending layers, respectively. Frog epidermis is ciliated in nearly all researched species until the tadpoles reach Gosner stages 25-30, when the cilia is lost bodily except for around the nose and eyes. Although not confirmed, scientists suspect that the mucociliary epithelium may play a role in sweeping microbes away

from the frog's body. Additionally, because the skin is the body's first line of immune defense against invaders, the stratum germinativum, or basal epithelium layer connected to the dermis, contains immune cells such as mast cells, macrophages, and lymphocytes as well as chromatophores and epithelial cells. The dermis has a top spongious dermal layer and a lower compact dermal layer. The spongious layer is mostly loose connective tissue and the lower compact layer bundles of collagen fibers with fibronectin filling in the gaps. The fibroblastic cells can be found in the lower compact layer and produce collagen to anchor the epithelium and dermis to the hypodermis beneath. Terrestrial amphibians have a non-cellular Eberth-Katschenko (EK) layer that separates the 2 layers of the dermis. The EK layer is found dorsally and mostly composed of glycoconjugates and glycosaminoglycans like hyaluronan and dermatan sulfate, which aid in water retention. In addition to immune cells mentioned previously, mucosal and granular glands are essential to the frogs' skin integrity because they maintain moisturization and permeability of the skin and provide an immune response by producing antimicrobial products, respectively (Varga et al., 2019).

Although no research has been conducted histologically on the skin system of *L.sylvatica*, the structure can easily be compared to a similar species, *Xenopus laevis*. Figure 3, shown below, identifies some labeled layers and components of skin previously discussed.

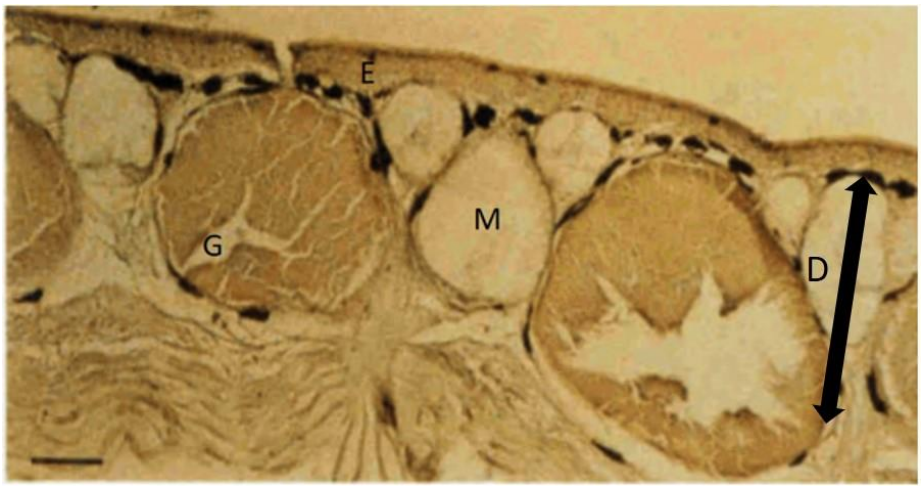

Figure 3. Immunohistochemical slide of *Xenopus laevis* skin. Labels are e, m, d, and g representative of the epidermis, mucous glands, dermis, and granular glands, respectively (Hauser et al. 1992).

Health and Harm of Epithelium of *L. sylvatica*

Toxicants can have negative impacts during two phases of an organisms life: (1) during organogenesis when the organs are being formed in development (effects are termed "organizational") and (2) after the organs are formed (effects are termed "activational", see Guillette et al., 1995). Table 2 displays some of these activational epithelial toxicants.

Table 2: A compiled list of substances with their respective damages to the epithelial system.

Substance	Effect	Source
Tobacco smoking	Premature skin aging via damaged collagen biosynthetic pathway	(Morita et al., 2009)
Cocaine	Levamisole-induced vasculitis and neutropenia	(Belfonte et al., 2012)
UV Radiation	Skin dehydration, epidermal hyperplasia, decrease of soluble type I collagen, skin cancer	(Tanaka et al., 2009)
Anabolic Steroids	Increased surface lipid cholesterol	(Király C.L.,1988)
Stress/Anxiety	Increased epithelial allergic histamine response	(Heffner et al., 2015)
Methamphetamine	Skin lesions with high risk of antibiotic-resistant bacterial contraction	(Cohen et al., 2007)

Purpose

The purpose of this study is to determine the effect of CBD intake on epithelial structure formation in different developmental stages of *Lithobates sylvatica*. This will be accomplished through histological evaluation of epithelium from tadpoles and frogs exposed and unexposed to the compound.

CHAPTER II

MATERIALS AND METHODS

Study Subjects

Animal Care

All animal husbandry, treatment, and collection protocols were approved by the Maryville College IACUC committee (see Appendix I). Twenty wood frog (*Lithobates sylvatica)* eggs were collected from 3 separate clutches on February 6, 2020 on private land in Walland, Tennessee. The water was 14°C at the time of collection, and over 5 hours the water temperature inside the collection buckets was increased to room temperature water (21°C) and the clutches were transferred into separate 10 gallon dechlorinated tanks filled with 20 tadpoles each. After transferal, it was determined via dissection microscope and Gosner staging guide (Gosner 1960) that the tadpoles were at stage 21. Tadpoles were maintained on a 14:10 photoperiod.

On February 27, 2020, tadpoles were collected from the same clutches in Walland, TN. Because outdoor temperature averaged 10.4°C during February, these tadpoles were much earlier in development than those in the lab. The tadpoles were allowed to acclimate to the temperature of the research lab prior to transferal into 2 new 10 gallon dechlorinated tanks. Twenty tadpoles were placed in a 10 gallon control tank while 23 were placed in another. Prior to the beginning of the experiment, all tadpoles were weighed and 2 were staged from each tank. The resident research lab tadpoles were determined to be stage 40 at

the beginning of the experiment while recently acquired tadpoles were stage 27. All specimens were weighed, and a t-test was performed to ensure there were no significant tank weight differences preceding the experiment.

CBD Treatment Preparation

Control frogs were fed Navisco Frog Brittle Powder. For the experimental group, CBD was integrated into the Navisco Frog Brittle Powder being fed. This was accomplished by weighing stage 21 tadpoles and acquiring a dosage of 15 mg/kg according to daily feeding amount (.5 g) and weight of all tadpoles in the tank. 55.2 mg CBD isolate was added to 80 g of Navisco Frog Brittle Powder along with approximately 250 mL of pure ethanol to dissolve the CBD fully into the powder and maintain an even dosage throughout the food. The CBD powder mixture was rotovapped at 142 RPM until all ethanol was eliminated and the food was completely dry.

After rotovapping, .5 g of CBD food and 3 mL of HPLC-grade methanol was added to 5 centrifuge tubes and centrifuged for 5 minutes at 7000 RPM. The supernatant was collected and placed into gas chromatography vials for HPLC evaluation. One of the vials was discarded due to large particle contamination, but the remaining 4 were placed in the HPLC unit. Each feeding was supposed to contain 345 µg of CBD, meaning that every vial in the HPLC unit needed to measure around 115 PPM to show that the CBD was evenly mixed throughout the food. All vials measured around 100 PPM, meaning that the CBD content was constant, but there was likely water still in the food particles, causing a small PPM difference on the HPLC readings. Both bags of food were stored in a 0°C fridge to prevent molding because water was in the CBD frog brittle and variables kept constant.

Exposure Period

To begin the experiment on March 2, 2020, a control tank from each stage of tadpoles was fed Navisco Frog Brittle Powder without CBD while experimental tanks of each stage were fed with Navisco Frog Brittle Powder containing CBD. Stage 27 tadpoles were fed .16 g while stage 40 tadpoles were fed .5 g. The tadpoles were allowed to eat ad libitum and 50% water changes were conducted once weekly. Stage 27 tadpoles' feeding amount increased as growth increased. A ramp made of ceramic plates was installed on March 10, 2020 to allow terrestrial transition. On March 16, 2020, the old tanks were removed and replaced for the fully metamorphosed frogs because there was progressive death in the ramp tanks and the small tadpoles were staged at stage 40 and fed .32 g daily. Two 10 gallon terrariums were constructed with 1 gallon of fresh, dechlorinated water and faux leaves as enrichment. The tanks were tilted on a small piece of wood so that there was a small water pool on one end and leaves with a small glass plate of food on the other. When the frogs metamorphosed, the food was made into a paste from the powder and placed in the shallow glass bowls of the terrariums. No casualties were recorded after the terrarium change. All found dead in tanks were removed, dissected, and kept for later evaluation in Bouin's fixative. The experiment was carried out for 16 days until the frogs were anesthetized, fixed, and prepared for histology.

Euthanization and Fixation

On March 19, 2020, the frogs and tadpoles were all individually weighed using a weigh boat and digital scale. Frogs and tadpoles were anesthetized using .5 grams of MS-222 in 500 mL of ddH_2O by placement into the anesthetizing agent in a small glass dish with a large beaker on top to prevent escape of the frogs. After all had been anesthetized, they were

dissected and fixed in Bouin's solution in a sterile cup to conduct histology on later. Tadpoles were staged under the dissection microscope and outliers in the frog and tadpole groups were staged and recorded as well. All samples were labeled appropriately and placed in the histology lab.

Histological Progression

Tissue Separation/Clearing

June 19, 2020, the fixed frogs and tadpoles were removed from Bouin's solutions temporarily and a liver lobe from each specimen was removed under dissection microscope with microdissection tools. After removal of the livers, the livers and bodies were separated in their respective sample collection cups, labeled, and cleared continuously with 70% ethanol until little to no Bouin's fixative remained in the tissues. To preserve time, groups of the frog and tadpole bodies were separated in multiple sterile urine collection cups so to speed the clearing process. Both livers and bodies were cleared nearly every day for 2 weeks to remove as much fixative as possible before the embedding process began.

Embedding

Once the fixative was cleared from the livers and bodies, the bodies and livers were arranged in plastic cassettes and labeled appropriately. Because the livers were at risk of falling through the holes of the cassettes, small envelopes constructed from Kimwipes were utilized to keep them secured inside. Cassettes were placed in first placed in 80% ethanol and moved to the respective clearing agents hourly as shown in Table 3. When placed in the waxes, cassettes were quickly removed from the pressure oven and returned to the next wax before adjusting the pressure appropriately. While the samples were under pressure in the

oven, metal base molds were fit with plastic cassette tops, labeled, and a drop of glycerol was added to the bottoms to prevent wax adhesion.

Table 3. Progression chart from respective hourly transfer of specimens to embedding in paraffin wax.

Product	Pressure	Time Exposed
80% ethanol	-	1 hour
95% ethanol	-	1 hour
100% ethanol	-	1 hour
100% ethanol	-	1 hour
Safeclear	-	1 hour
Safeclear 2	-	1 hour
Wax I	12 in. Hg	1 hour
Wax II	15 in. Hg	1 hour
Wax III	21 in. Hg	1 hour
Wax IV	25 in. Hg	1 hour

After Wax IV, the cassettes were placed in a heated paraffin wax pot to prevent tissue hardening and were removed to orient the specimens into paraffin wax blocks. Paraffin was added continuously as the blocks dried to prevent air bubbles and wax sinking.

Sectioning and Preparation of Slides

The wax blocks containing study bodies and livers were cut into pyramids with a razor blade, with the organ/site of interest at the top of the pyramid. The blocks were placed on the microtome and cut into ribbons 12 µm thick. The ribbons were placed on an adjacent tray from beginning to end and were cut using a razor blade to fit multiple ribbons on microscope slides. The cut ribbons were floated in a warmed water bath that contained a pinch of gelatin and mounted on labeled slides. The slides were placed on the slide warmer for a day to dry for staining.

Staining

Staining procedures were followed according to Figure 4 under a fume hood. Because the hematoxylin stain was very dark, slides were only submerged for 3.5 minutes instead of

4. After the staining procedure was finished, the slides were completed by application of Permount and applying a cover slip over the tissue. Slides were left under the fume hood for 24 hours to allow the Permount to fully dry before evaluating the tissues.

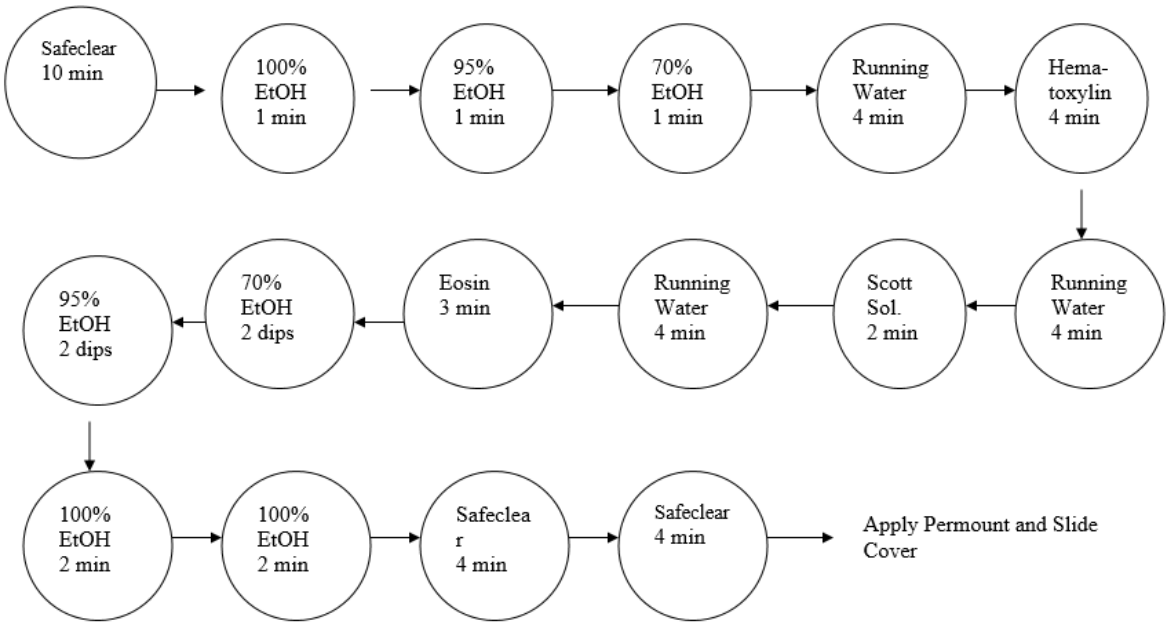

Figure 4. Staining procedure followed for histology evaluation (Crain 2020).

Quantitative Measurements

Endpoints

Length, width, and density of stratified squamous cells in the epithelium were recorded, as well as mucosal secretory cell length and width. Diameters of the secretory duct openings were recorded. Fifteen measurements were taken of each cell and 5 ducts were measured per specimen. Cell measurements of the epithelial tissue were conducted at the beginning of the eye for bodily study figures. A 0.1 mm stage micrometer was calibrated to the microscope and used to calculate length and width of each cell type. After all measurements had been recorded, photos of the structures were taken with a Nikon camera attachment and labeled. Additionally, although not quantified, the activity level was noted in

the animals prior to euthanasia. Labeled structures quantified in results for both frog and tadpole specimens can be found in Figures 5 and 6.

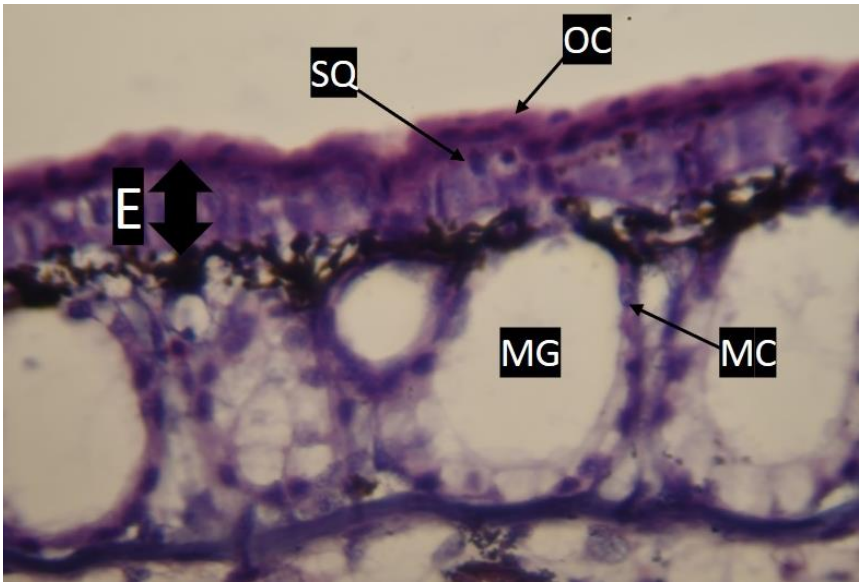

Figure 5. Labeled, stained slide at 400x magnification of a wood frog's epithelial system. Epidermis layer (E), squamous cells (SQ), outer cells (OC), mucous gland (MG), and mucosal cells (MC) are shown.

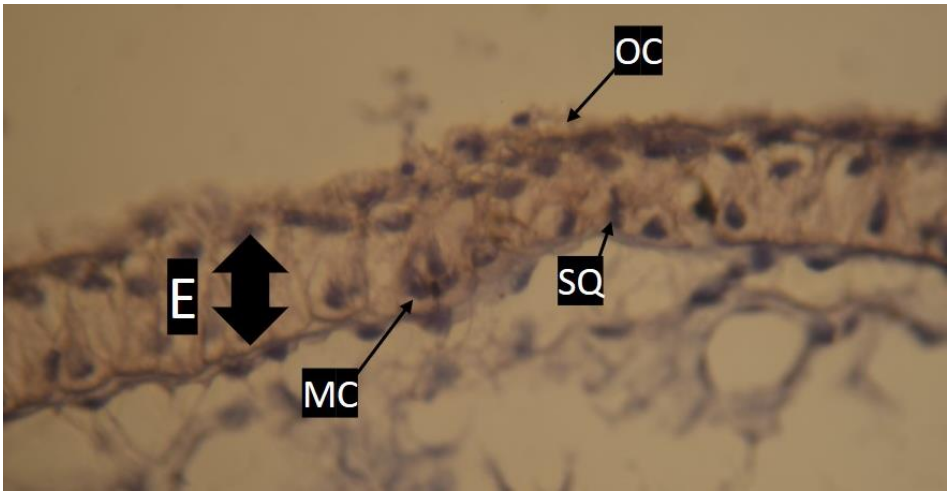

Figure 6. Labeled, stained slide at 400x magnification of a wood frog tadpole's epithelial system. Epidermis layer (E), squamous cells (SQ), outer cells (OC), mucous gland (MG), and mucosal cells (MC) are shown.

Statistical Analyses

The statistical features conducted in the experiment were all found using Excel. Two-tailed t-tests were utilized to determine significance between factors and averages as well as standard error were calculated for all cells and measurements. This data was compiled into bar graphs with standard error accounted for and statistical significance was noted. Averages of each specimen cell type and characteristic were used for the individual cell. T-tests were performed on both control and experimental groups in an effort to discover statistical significance between cell measurements and were also utilized to ensure that there were no significant weight differences between either group.

CHAPTER III

RESULTS

Observations

Though not quantified, both tadpoles and frogs exposed to CBD were less reactive than control frogs/tadpoles when removing them from their respective tanks. Their movements were also slower and more delayed than control frogs and tadpoles. Due to time constraints, these could not be measured appropriately.

After all frogs and tadpoles had been anesthetized and observed for gross physical appearance, it was determined that the majority of frogs had fully metamorphosed according to Gosner staging, with the exception of 2 control frogs being stage 45, 2 exposed frogs being stage 45 and another 2 exposed frogs stage 44. Tadpoles were Gosner stage 40 with 1 exposed tadpole being stage 25. Two large spinal abnormalities were found in the exposed tadpole group, shown in Figure 7, along with the noticeably smaller tadpole staged 25.

Figure 7. Photograph of spinal abnormalities and small tadpole found in exposed tadpole tank.

At the end of the experiment, control frogs and exposed frogs showed no significant difference in their weights ($p=0.578$), however tadpoles exposed to CBD weighed significantly more than those who were not exposed ($p<0.01$).

Histology

After histological evaluation, it was noted that the frog epidermis was filled with mainly cuboidal cells with some columnar, while the tadpole epidermis was predominantly columnar with some cuboidal cells in areas of cell division. Figures 8 and 9 are labeled accordingly to their respective group. Because mucosal glands and cells had not been developed, there are less specimens accounted for tadpole groups.

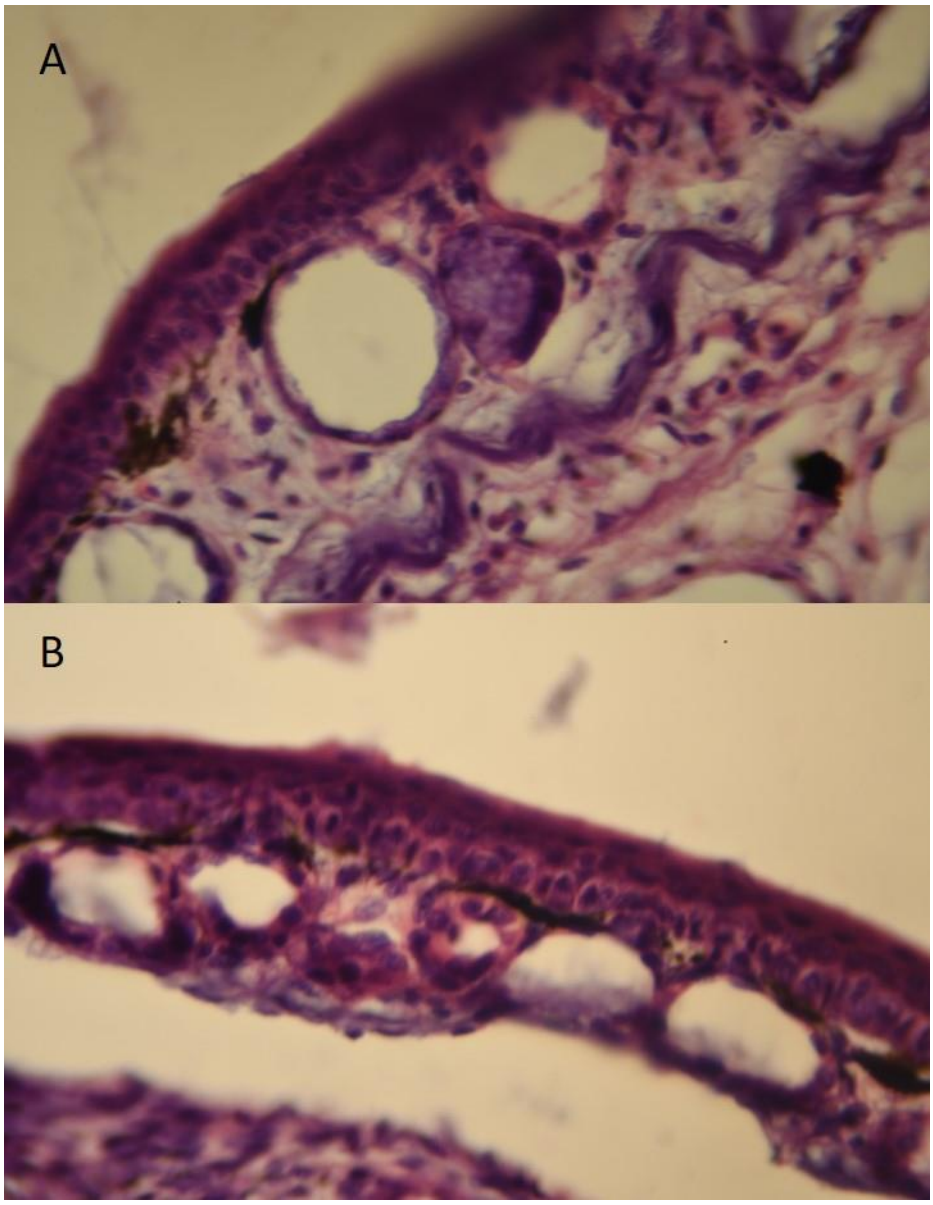

Figure 8. Photographs of control (A) and exposed (B) frog epithelial systems at 400x magnification.

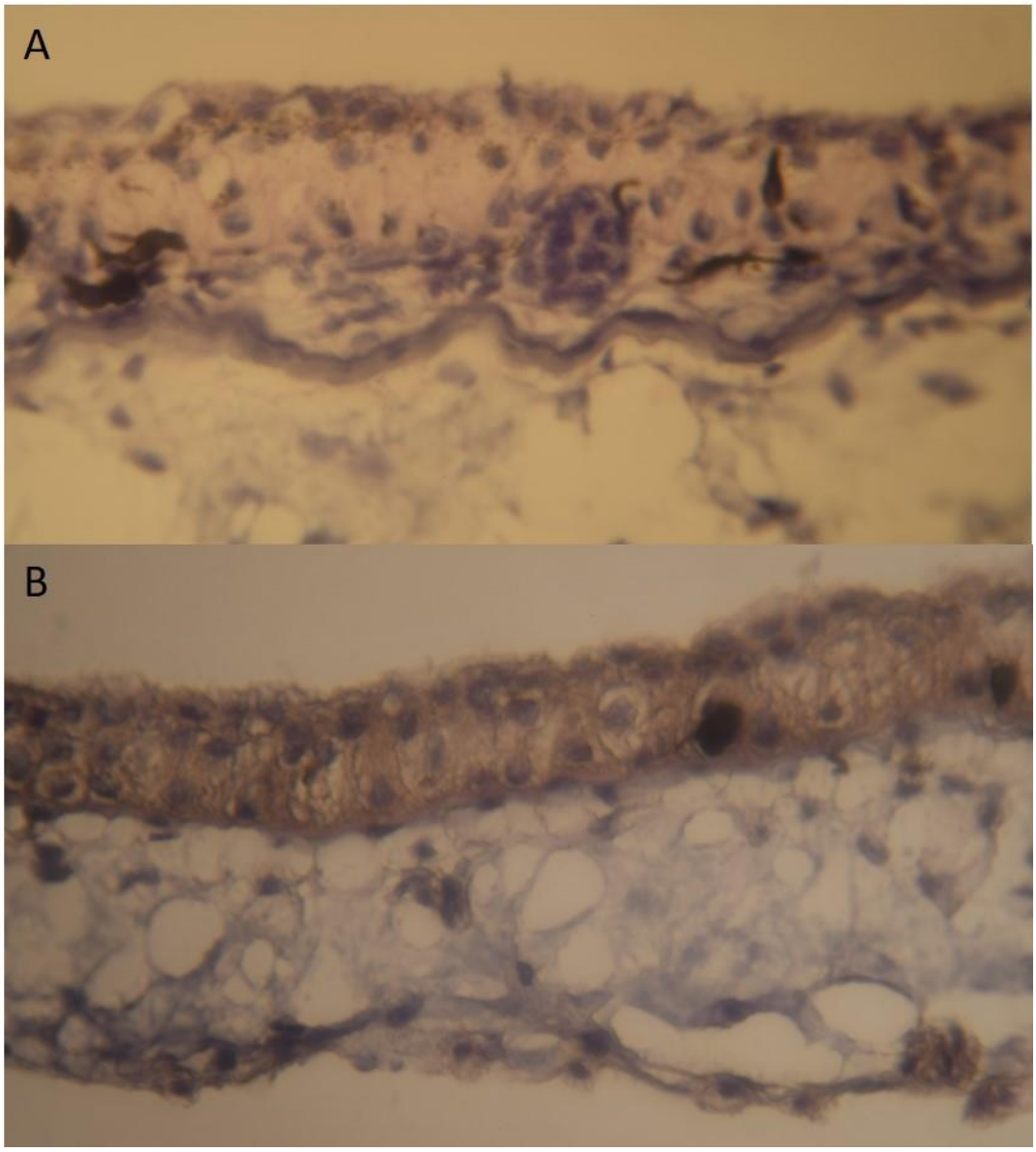

Figure 9. Photograph of control (A) and exposed (B) tadpole epithelial systems at 400x magnification. Areas of cell division in the lower epidermis to form mucosal and other glands can be seen.

Morphometrics

There was no significant difference in epidermal thickness between the frog control and exposed groups (p=0.604; Fig. 10A). Tadpoles also had no significant difference (p=0.113; Fig. 11A). Densities were not statistically significant for frogs' exposed (p=0.604;

Fig. 10B), but exposed tadpoles showed a significantly lower density than the control ($p<0.001$; Fig. 11B). There was no significant difference between frog control and exposed squamous cell height ($p=0.664$; Fig. 10C) or width ($p=0.742$; Fig. 10C). Exposed tadpoles' height was found to be significantly shorter ($p<0.001$; Fig.11C) than the control, but width did not change significantly ($p=0.674$; Fig. 11C). Frogs' exposed group showed a significantly shorter outer cell height ($p<0.001$; Fig. 10D) and larger width ($p<.001$; Fig. 10D) when compared to the control. Exposed tadpoles showed no difference between outer cell height ($p=0.117$; Fig. 11D) and width ($p=0.772$; Fig. 11D). Frog mucosal cells showed no significant difference in regard to exposed height ($p=0.693$; Fig. 10E) and width ($p=0.800$; Fig. 10E). Exposed tadpoles' mucosal cells were shorter ($p<.001$; Fig. 11E) and wider ($p<0.001$; Fig. 11E) than the control, however. Lastly, no significant differences were found regarding the diameter of mucous glands for exposed frogs versus the control ($p=0.531$; Fig. 10F). Figures 10 and 11 contain all figures referenced above for both tadpole and frog specimens.

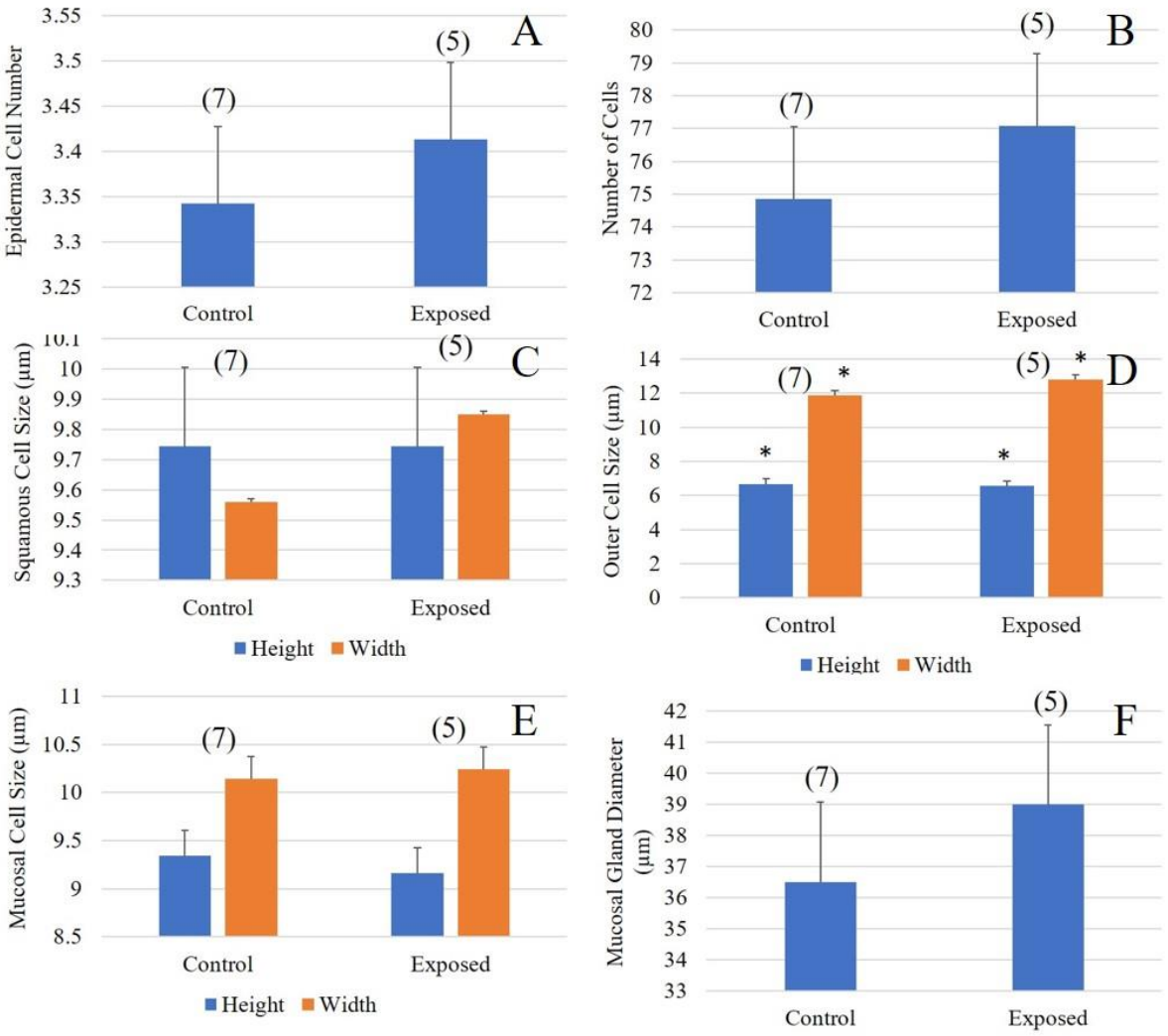

Figure 10. Bar graphs displaying newly metamorphosed frog control and exposed averages (±SE) for epidermal thickness (A), density (B), squamous height and width (C), outer cell height and width (D), mucosal cell height and width (E), and mucosal gland diameter (F). Bars with * above them represent statistical significance at $p<0.05$.

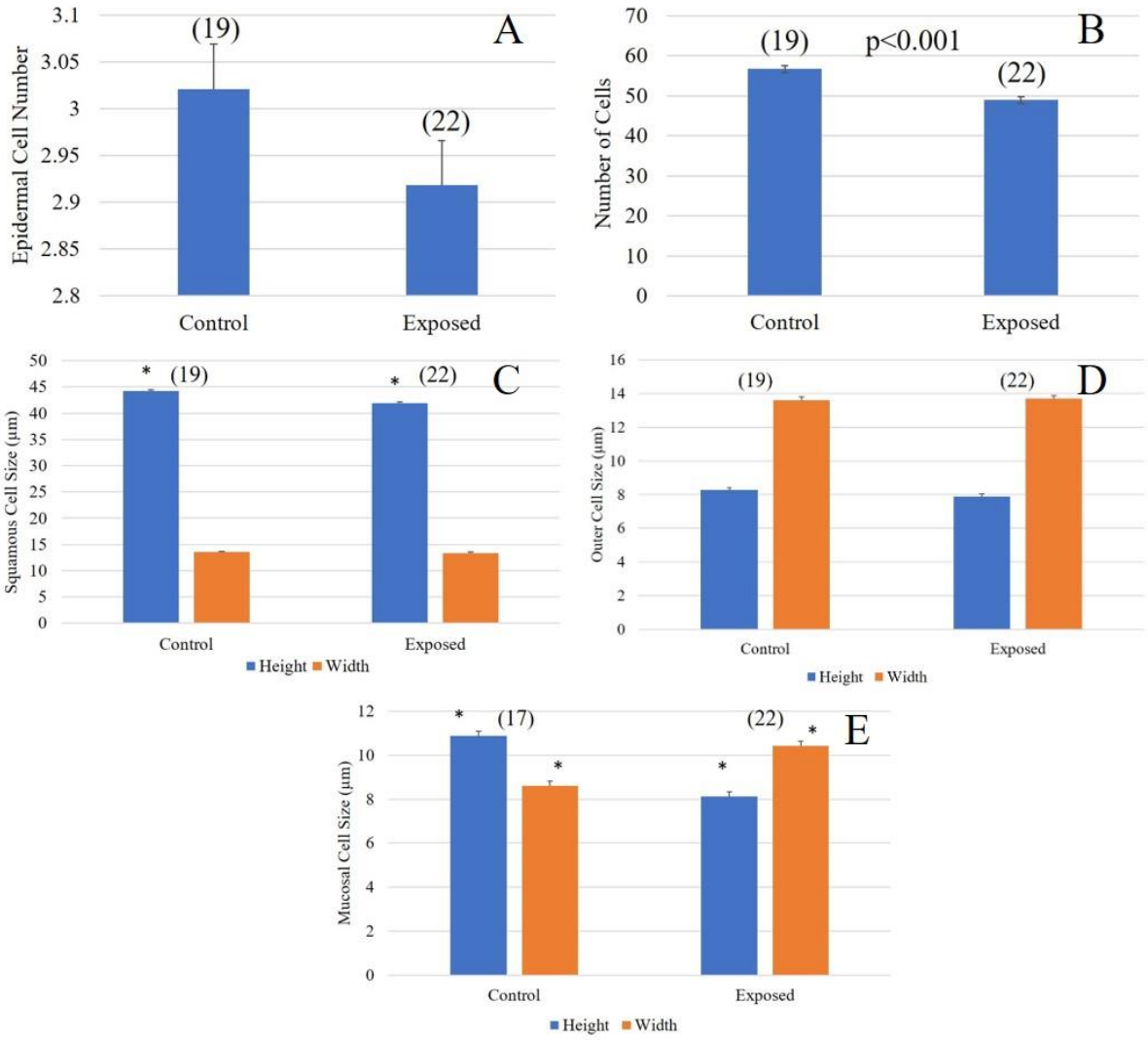

Figure 11. Bar graphs displaying tadpole control and exposed averages (+SE) for epidermal thickness (A), density (B), squamous height and width (C), outer cell height and width (D), and mucosal height and width (E). Bars with * above them represent statistical significance at $p<0.05$.

CHAPTER IV

DISCUSSION

Quantitatively, results found that both tadpoles and frogs have some significant differences between the control and exposed groups. Exposed tadpoles experienced a lower density of epithelial cells, shorter squamous cell height, and shorter and wider mucosal cell heights when compared to controls. Exposed frogs experienced a wider and shorter outer cell size than the controls. These results suggest that cannabidiol influenced the development of *L. sylvatica* epithelium.

Exposed tadpoles' epithelial cells were significantly less dense than those of the control. Prior studies examined CBD's inhibitory effects on cell division and upregulation of apoptotic factors, as cannabidiol increases ROS species in gastric cancerous cells among others and downregulates genes needed to proliferate and divide cells (Zhang et al., 2019). Further research shows that it prevents invasion of growing cancer cells into neighboring tissues (Ramer et al., 2011). Cannabidiol's anti-invasive action along with the compound's cyclic and apoptotic regulation properties could all be probable causes for this finding.

As for the exposed tadpoles experiencing shorter squamous with shorter and wider mucosal cells, as well as the shorter and wider cell size of exposed frogs, other researchers have found similar results. According to one study, cannabidiol significantly reduces Jurkat T cell size. This study used leukemic cells to demonstrate that reduced cell size persists after

CBD treatment and normal-sized cell number decreases (Kalenderoglou et al., 2017). Because cannabidiol impacts gene expression and regulation, especially factors in the cell cycle in G0-G1 phase, it can have a significant influence on the growth and size of cells. The commitment point of the G0 to G1 transition is crucial to cell size and growth (Lea et al., 2003). Cannabidiol administration has been associated with a higher percentage of cells in the G2 and M phases than the G0 and G1 phases in neural cells as well because of its arresting properties (Al-Ghezi et al., 2019). These cell cycle regulatory factors in cannabidiol are all potential connections to smaller cell size.

Although not quantified, it was very noticeable that exposed frogs and tadpoles were much less active than the controls and controls were much more reactive than exposed subjects. Prior research has found that cannabidiol "reduces the action potential firing of striatal neurons" as well as inhibits persisting currents of sodium channels (Patel et al., 2016). Sodium channels are very important in the firing of neurons to produce muscular movements. Cannabidiol is currently being used in trials to control incurable epileptic seizures in adults and children and has reduced seizure frequency while having relatively no side effects (Devinsky et al., 2016). These seizures are related to the reduced threshold for action potentials, causing sodium channels to fire often and consistently, causing persistent nerve and muscle stimulation (Chen et al., 2002). Inhibition of sodium potential currents is a potential factor in this activity reduction seen in the tadpoles and frogs. Future studies should examine these effects by using grid crossings. Previously, this has been a very reliable helpful technique for measuring activity level of animals of the same age (Vila et al., 2004).

Another observed effect the exposed tadpoles experienced was severe spinal deformities in 2 of 23 specimens and one tadpole that was 15 stages behind the rest.

Interestingly, cannabinoids are commonly used in reduction of inflammation for mature spinal cord injuries because of their anti-inflammatory regulation (Adhikary et al., 2011). They have been used to reduce injury time and increase locomotion during recovery (Kwiatkoski et al., 2011). One study does show, however, that CBD does affect the chain packing for the lipid bilayer because of its insertion locations under the lipid heads (Watkins et al., 2020). This could potentially affect the composition and development of the myelin membrane and impact its efficacy against invaders and injury (Svennerholm et al., 1992). Additionally, other studies have found deformities in fins, swim bladders, and behavior in other animal species (Carty et al., 2017). Regarding size, however, prior research has found no significant difference in growth and growth hormone levels of those who ingest CBD compared to those who have not (Zuardi et al., 1993). Unfortunately, there have been very few studies that evaluate developmental impact of CBD, so these observations cannot be fully understood.

In conclusion, this study shows evident developmental impacts on skin epithelium from cannabidiol exposure in wood frogs and wood frog tadpoles. Quantifiable evidence is supported by findings in literature and previous studies, however visual deformities did not have enough research to adequately evaluate. These developmental effects can be a cause for concern if ingested by pregnant women or small children and should be a focus point for research. Because CBD is not a regulated compound by the FDA, it is important to continue developmental studies to determine potential defects and establish safe administration practice to humans and animals.

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:
Bryan Crouser
Student Email Address:
bryan.crouser@my.maryvillecollege.edu
Date:
2/11/20
Senior Study Advisor:
Dr. Crain
Species to be used:
Rana sylvatica (Wood Frogs)
Age of animals:
Early stage 26-46 tadpoles
Number of animals in study:
100
Duration of study:
Present-May 31 2020
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.
Bryan Crouser	423-762-5807	423-762-5807	423-715-9335
D. Andrew Crain	8238	292-8737	

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.
The animal will be anesthetized using Euthanasia via MS222 500mg/L which will be injected using a syringe through the pectoral muscle. Time will be given for the animals will to become fully unconcious. Then the animals will be disected in which the liver and the thyroid will both be isolated. These organs will then go through the process of histology.

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

Maryville College IACUC Approval Number: 202001
Date Approved: 2/24/20
Signed:

WORKS CITED

Adhikary S., Li H., Heller J., Skarica M., Zhang M., Ganea D., Tuma R.F. (2011). Modulation of inflammatory responses by a cannabinoid-2-selective agonist after spinal cord injury. *Journal of Neurotrauma*, 28(12):2417-2427.

Batalla A., Janssen H., Gangadin S.S., Bossong M.G. (2019). The potential of cannabidiol as a treatment for psychosis and addiction: who benefits most? A systematic review. *Journal of Clinical Medicine, 8*(7):1058.

Bee M.A. (2007). Selective phonotaxis by male wood frogs (*Rana sylvatica*) to the sound of a chorus. *Behavioral Ecological Sociobiology, 61*:955-966.

Belfonte C.D., Shanmugam V.K., Kieffer N., Coker S., Boucree S., Kerr G. (2012). Levamisole-induced occlusive necrotizing vasculitis in cocaine abusers: an unusual cause of skin necrosis and neutropenia. *Internal Wound Journal*, 10(5):590-596.

Bergamuschi M.M., Costa Quieroz R.H., Crippa J.A.S., Zuardi A.W. (2011). Safety and side effects of cannabidiol, a *Cannabis sativa* constituent. *Current Drug Safety, 6*(4):1-13.

Bullen C. (2008). Impact of tobacco smoking and smoking cessation on cardiovascular risk and disease. *Expert Review of Cardiovascular Therapy, 6*(6):883-895.

Burstein S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorganic and Medicinal Chemistry, 23*:1377-1385.

Carty D.R., Thornton C., Gledhill J.H., Willet K.L. (2017). Developmental effects of cannabidiol and Δ^9-tetrahydrocannabinol in zebrafish. *Toxicological Sciences*, 162(1):137-145.

Chen A., Ashburn M.A. (2015). Cardiac effects of opioid therapy. *Pain Medicine, 16*(1):S27-S31.

Chen C., Bharucha V., Chen Y., Westenbroek R.E., Brown A., Malhotra J.D., Jones D., Avery C., Gillespie III P.J., Kazen-Gillespie K.A., Kazarinova-Noyes K., Shrager P., Saunders T.L., Macdonald R.L., Ransom B.R., Scheuer T., Catterall W.A., Isom L.L. (2002). Reduced sodium channel density, altered voltage dependence of inactivation, and increased susceptibility to seizures in mice lacking sodium channel β2-subunits. *PNAS*, 99(26):17072-17077.

Cohen A.L., Shuler C., McAllister S., Fosheim G.E., Brown M.G., Abercrombie D., Anderson K., McDougal L.K., Drenzek C., Arnold K., Jernigan D., Gorwitz R. (2007). Methamphetamine use and methicillin-resistant *Staphylococcus aureus* skin infections. *Emergency Infection Discourse*, 13(11):1707-1713.

Corroon J., Knight R. (2018). Regulatory status of cannabidiol in the United States: a perspective. *Cannabis Cannabinoid Research, 3*(1):190-194.

Crain D. (2020). Animal Physiology Laboratory Manual. Maryville College.

Devinsky O., Marsh E., Friedman D., Thiele E., Laux L., Sullivan J., Miller I., Flamini R., Wilfong A., Filloux F., Wong M., Tilton N., Bruno P., Bluvstein J., Hedlung J., Kamens R., Maclean J., Nangia S., Singhal N.S., Wilson C.A., Patel A., Cilio M.R. (2015). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurology, 15*: DOI: 10.1016/S1474-4422(15)00379-8.

Dieni C.A., Storey K.B. (2008). Regulation of 5'-adenosine monophosphate deaminase in the freeze tolerant wood frog, *Rana sylvatica*. *BMC Biochemistry, 9*(12): DOI: 10.1186/1471-2091-9-12.

Dodd Jr. C.K. 2004. The amphibians of Great Smoky Mountains National Park. Knoxville (TN): University of Tennessee Press.

Fischer B., Kuganesan S., Gallassi A., Malcher-Lopes R., Van Den Brink W., Wood E. (2015). Addressing the stimulant treatment gap: a call to investigate the therapeutic benefits potential of cannabinoids for crack-cocaine use. *International Journal of Drug Policy, 26*:1177-1182.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.

Guillette Jr. L.J., Crain DA, Rooney A.A., Pickford D.B. (1995). Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environmental Health Perspective*, 103(7):157-164.

Hathcock J. (2001). Dietary supplements: how they are used and regulated. *Journal of Nutrition*, 131(3):1114S-1117S.

Hauser F., Roeben C., Hoffmann W. (1992). xP2, a new member of the p-domain peptide family of potential growth factors, is synthesized in *Xenopus laevis* skin. *The Journal of Biological Chemistry, 267*(2):14451-14455.

Heffner K.L., Kiecolt-Glaser J.K., Glaser R., Malarkey W.B., Marshall G.D. (2014). Stress and anxiety effects on positive skin test responses in young adults with allergic rhinitis. *Annals of Allergy, Asthma & Immunology*, 113(1):P13-18.

Hernandez R.K., Werler M.M., Romitti P., Sun L., Anderka M. (2018). Nonsteroidal antiinflamatory drug use among women and the risk of birth defects. *American Journal of Obstetrics and Gynecology, 206*(3):228.e1-228.e8.

Jadoon K.A., Tan G.D., O'Sullivan S.E. (2017). A single dose of cannabidiol reduces blood pressure in healthy volunteers in a randomized crossover study. *JCI Insight, 2*(12); e93760.

Jenkins K.J., Correa A., Feinstein J.A., Botto L., Britt A.E., Daniels S.R., Elixson M., Warnes C.A., Webb C.L. (2007). Noninherited risk factors and congenital cardiovascular defects: current knowledge. *Circulation, 115*:2995-3014.

Johansen K., Hanson D. (1968). Functional anatomy of the hearts of lungfishes and amphibians. *American Zoologist, 8*:191-210.

Kalenderoglou N., Macpherson T., Wright K.L. (2017). Cannabidiol reduces leukemic cell-size – but is it important? *Frontiers in Pharmacology,* 8:144.

Király C.L. (1988). Androgenic-anabolic steroid effects on serum and skin surface lipids, on red cells, and on liver enzymes. *International Journal of Sports Medicine*, 9(4):249-252.

Kwiatkoski M., Guimarães F.S., Del-Bel E. (2011). Cannabidiol-treated rats exhibited higher motor score after cryogenic spinal cord injury. *Neurotoxicity Research*, 21:271-280.

Lea N.C., Orr S.J., Stoeber K., Williams G.H., Lam E.W.F., Ibrahim M.A.A., Mufti G.J., Thomas N.S.B. (2003). Commitment point during G0→G1 that controls entry into the cell cycle. *Molecular and Cellular Biology*, 23(7):2351-2361.

Lee W.S., Erdelyi K., Matyas C., Mukhopadhyay P., Varga Z.V., Liaudet L., Haskó G., Čiháková D., Mechoulam R., Pacher P. (2016). Cannabidiol limits t cell-mediated

chronic autoimmune myocarditis: implications to autoimmune disorders and organ transplantation. *Molecular Medicine, 22*:136-146.

McNally J.D., Wu S.B., Sturgeon C.M., Storey K.B. (2002). Identification and characterization of a novel freezing-inducible gene, *li16*, in the wood frog *Rana sylvatica*. *FASEB Journal, 16*(8):902-904.

Mead A. (2017). The legal status of cannabis (marijuana) and cannabidiol (CBD) under U.S. law. *Epilepsy and Behavior, 70*(B):288-291.

Meeks D.E., Nagel J.W. (1973). Reproduction and development of the wood frog, *Rana sylvatica*, in eastern Tennessee. *Herpetologica, 29*:188-191.

Mohun T.J., Leong L.M., Weninger W.J., Sparrow D.B. (2000). The morphology of heart development in *Xenopus laevis*. *Developmental Biology, 218*:74-88.

Morita A., Torii K, Maeda A., Yamaguchi Y. (2009). Molecular basis of tobacco smoke-induced premature skin aging. *Journal of Investigative Dermatology Symposium Preceedings,* 14(1):53-55.

Myers J. (2003). Exercise and cardiovascular health. *Circulation, 107*(1):e2-e5.

Nissen S.E. (2006). ADHD drugs and cardiovascular risk. *New England Journal of Medicine, 354*:1445-1448.

Patel R.R., Barbosa C., Brustovetsky T., Brustovetsky N., Cummins T.R. (2016). Abberant epilepsy-associated mutant $Na_v1.6$ sodium channel activity can be targeted with cannabidiol. *Brain: A Journal of Neurology,* 139(8): 2164-2181.

Perez A., Mazerolle M.J,. Brisson J. (2013). Effects of exotic common reed (Phragmites australis) on wood frog (Lithobates sylvaticus) tadpole development and food availability. *Journal of Freshwater Ecology, 28*(2):165-177.

Pryce G., Riddall D.R., Selwood D.L., Giovanni G., Baker D. (2014). Neuroprotection in experimental autoimmune encephalomyelitis and progressive multiple sclerosis by cannabid-based cannabinoids. *Journal of Neuroimmune Pharmacology, 10*:281-292.

Ramer R., Merkord J., Rohde H., Hinz B. (2010). Cannabidiol inhibits cancel cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1. *Biochemical Pharmacology*, 79(7): 955.

Rajesh M., Mukhopadhyay P., Bátkai S., Patel V., Saito K., Matsumoto S., Kashiwaya Y., Horváth B., Mukhopadhyay B., Becker L., Haskó G., Liaudet L., Wink D.A., Veves A., Mechoulam R., Pacher P. (2010). Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. *Journal of the American College of Cardiology, 56*(25):2115-2125.

Regosin J.V., Windmiller B.S., Reed J.M. (2003). Terrestrial habitat use and winter densities of the wood frog (*Rana sylvatica*). *Journal of Herpetology, 32*(2):390-394.

Relyea R.A. (2002). Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. *Ecological Monographs, 72*(1):77-93.

Schwartz B.G., Rezkalla S., Kloner R.A. (2010). Cardiovascular Effects of Cocaine. *Circulation, 122*(24):2558-2569.

Sedmera D., Reckova M., deAlmeida A., Sedmerova M., Biermann M., Volejnik J., Sarre A., Raddatz E., McCarthy R.A., Gourdie R.G., Thompson R.P. (2002). Functional and morphological evidence for a ventricular conduction system in zebrafish and *Xenopus* hearts. *American Journal of Physiological Heart Circular Physiology, 284*:H1152-H1160.

Shannon S., Lewis N., Lee H., Hughes S. (2019). Cannabidiol in anxiety and sleep: a large case series. *Permanente Journal, 23*:18-014.

Shelton G., Boutilier R.G. (1982). Apnoea in amphibians and reptiles. *Journal of Experimental Biology, 100*:245-273.

Sinning A.R. (1998). Role of Vitamin A in the formation of congenital heart defects. *The Anatomical Record, 253*:147-153.

Stanley C.P., Hind W.H., O'Sullivan S.E. (2012). Is the cardiovascular system a theurapeutic target for cannabidiol?. *British Journal of Pharmacology, 75*(2):313-322.

Stergiopoulos K., Brennan J.J., Mathews R., Setaro J.F., Kort S. (2008). Anabolic steroids, acute myocardial infarction and polycythemia: a case report and review of the literature. *Vascular Health Risk Management, 4*(6):1475-1480.

Svennerholm L., Boström K., Fredman P., Jungbjer B., Månsson J.E., Rynmark B.M. (1992). Membrane lipids of human peripheral nerve and spinal cord. *Biochimica et Biophysica Acta (BBA) – Lipids and Lipid Metabolism*, 1128(1):1-7.

Tanaka M., Koyama Y., Nomura Y. (2009). Effects of collagen peptide ingestion on UV-B-induced skin damage. *Bioscience, Biotechnology, and Biochemistry*, 73(4):930-932.

Thomas G., Kloner R.A., Rezkella S. (2014). Adverse cardiovascular, cerebrovascular, and peripheral vascular effects of marijuana inhalation: what cardiologists need to know. *The American Journal of Cardiology, 113*(1):187-190.

Trauth S.E., McCallum M.L., Cartwright M.E. (2000). Breeding mortality in the wood frog, *Rana sylvatica* (anura: ranidae), from Northcentral Arkansas. *Journal of the Arkansas Academy of Science, 54*:154-156.

Varga J.F.A., Bui-Marinos M.P., Katzenback B.A. (2019) Frog skin innate immune defenses: sensing and surviving pathogens. *Frontiers in Immunology, 9*: DOI: 10.3389/fimmu.2018.03128.

Vila J.L., Philpot R.M., Kirstein C.L. (2004). Grid crossing: inability to compare activity levels between adolescent and adult rats. *Annals of the New York Academy of Science*, 1021(1):418-421.

Watkins A.R., Phaterpekar T., Ruben P.C., Thewalt J.L. (2020). Cannabidiol affects chain packing in lipid membranes. Biophysical Journal 118(3):389A.

Watkins T.B., Vraspir J. (2005). Both incubation temperature and posthatching temperature affect swimming performance and morphology of wood frog tadpoles (*Rana sylvatica*). *Physiological and Biochemical Zoology*, *79*(1):140-149.

Watt G., Karl T. (2017). *In vivo* evidence for therapeutic properties of cannabidiol (CBD) for Alzheimer's Disease. *Frontier Pharmacology, 8*:20.

Zhang X., Qin Y., Pan Z., Li M., Liu X., Chen X., Qu G., Zhou L., Xu M., Zheng Q., Li D. (2019). Cannabidiol induces cell cycle arrest and cell apoptosis in human gastric cancer SGC-7901 cells. *Biomolecules*, 9:302.

Al-Ghezi Z.Z., Miranda K., Nagarkatti M., Nagarkatti P.S. (2019). Combination of cannabinoids, Δ9-tetrahydrocannabinol and cannabidiol, ameliorates experimental multiple sclerosis by suppressing neuroinflammation through regulation of miRNA-mediated signaling pathways. *Frontiers in Immunology*, 10:1921.

Zuardi A.W., Guimarães F.S., Moreira A.C. (1993). Effect of cannabidiol on plasma prolactin, growth hormone, and cortisol in human volunteers. *Brazilian Journal of Medical and Biological Research*, 26(2): 213-217.