INVESTIGATION OF ALTERED BONE DEVELOPMENT CAUSED BY ANTICONVULSANT DRUG THERAPY IN *RANA SYLVATICA* TADPOLES

A Report of a Senior Study

by

Hillary Summers Brown

Major: Biology

Maryville College

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Date Approved _____, by_____
Faculty Supervisor

Date Approved _____, by_____
Editor

ABSTRACT

Patients receiving anti-epileptic drug therapy have shown decreased bone density possibly through the mechanism of hepatic cytochrome enzyme induction reducing calcium and phosphorus absorption in the intestines. This results in hypocalcemia and then hyperparathyroidism leading to reduced bone mineralization. This study used an amphibian model (*Rana sylvatica*) to investigate the effects of the anti-convulsant drug carbamazepine (CBZ) exposure dosed during two stages of metamorphic osteogenesis (early-dosed stage 29 and late-dosed stage 35). Carbamazepine did not influence humerus length in early groups (control 2.60 mm, CBZ 2.84mm, p=0.50) or late groups (control 3.18 mm, CBZ 3.20 mm, p=0.95), diameter in early (control 0.19mm, CBZ 0.23mm, p=0.42) or late (control 0.27mm, CBZ 0.25mm p=0.34) and percent ossification in early (control 39.3% CBZ, 48.2%, p=0.75) or late (control 49.1% and CBZ 28.7% P=0.33). The stage of dosage did not influence length (early 2.76mm, late 3.19mm p=0.060), diameter (early 0.217mm late 0.256mm p=0.065) or percent ossification (early 44.6% late 36.3% mm p= 0.59) either. Hyperparathyroidism, a side-effect of antiepileptic drug use, does not affect amphibian bone likely because of retained calcium control in the pituitary and paraphysis, and in that respect, amphibians are not a good model for bone osteogenesis.

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ACKNOWLEDGMENTS

First, I would like to thank my supervisor, Dr. Crain, for guiding me throughout this study, offering advice and criticism that have been invaluable. I would also like to extend my gratitude to Dr. Swann for providing her guidance in interpreting my histology slides.

CHAPTER I

Introduction

Anticonvulsant drugs lower bone density in adults being treated for epilepsy, as cases of osteomalacia, osteoporosis, and secondary hyperparathyroidism occur in patients on mono or poly-anticonvulsant drug therapy (Ray et al. 2002). In one study, patients being treated for epilepsy had bone densities at least 10 arbitrary density units lower than the control groups when scanned at the heel and at least 5 units lower when scanned at the elbow (Linde et al. 1971). The control groups consisted of non-epileptic psychiatric patients residing in the same complex and a group of normal healthy individuals, and no significant difference was found between the bone densities of the two control groups. These bone density decreases do not appear to be due to seasonal factors, and only influenced by the anticonvusant drugs (Linde et al. 1972). Such bone density decreases increase the incidence of skeletal fractures, with 28.6% of patients on one and 48.7% of patients on two or more anticonvulsant drugs suffered skeletal fractures during and after the period of treatment (Ray et al. 2002).

Epileptic patients are already at high risk for fractures because of other factors such as lack of exercise in the form of weight bearing activities and exposure to sunlight. Institutionalized patients usually have lower vitamin D levels than those of people living

at home due simply to the lack of variety in routines and diets in psychiatric facilities (Harrington & Hodkinson 1987). These factors can reduce bone strength by limiting deposition of calcium due to lack of vitamin D and by limiting bone growth and repair. Vitamin D is an essential component to bone ossification and calcification. Before it is used, vitamin D undergoes two hydroxylation reactions to become calcitriol (1,25 dihydroxyvitamin D_3). It then functions as a hormone by increasing the flow of calcium into the bloodstream by promoting the absorption of calcium and phosphorous from food and the reabsorption of calcium in the kidneys (see Figure 1 based on Guyton and Hall 2006). In this manner, calcium is made available for cellular uses, including bone formation

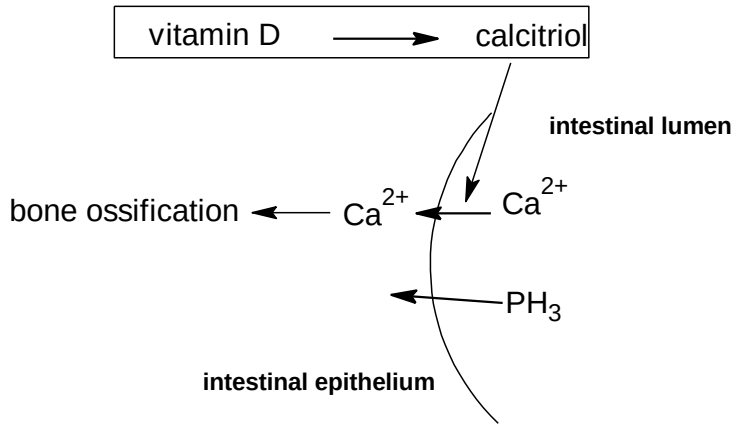

Figure 1: The actions of vitamin D on calcium and phosphorous absorption from the intestinal lumen resulting in bone ossification without the effects of anticonvulsant drugs.

Mechanism of action

Studies on the affects of these drugs have proposed a mechanism by which anticonvulsant drugs reduce bone density in patients. For the last two decades, scientists have known that long term- anticonvulsant drug therapy induces hepatic cytochrome enzymes that reduce the availability of vitamin D for renal hydroxylation (Harrington and Hodkinson 1987, Dent et al. 1970, and Linde et al. 1971). Recently, a specific hepatic microsomal enzyme, CYP3A4, has been implicated in this pathway (Xu et al. 2006). The CYP3A4 enzyme catalyzes the further hydroxylation of Vitamin D and, therefore, inactivates it. In this way, vitamin D, which is crucial for the regulation of calcium in the body, does not form calcitriol (1,25-Dihydroxyvitamin D_3;see Figure 2 based on Guyton and Hall). Calcitriol, which allows for the absorption of calcium and phosphorus from the intestines, plays an important role in bone growth and repair. In some cases however, supplemented vitamin D had no effect on the bone density of patients on anticonvulsant drug therapy despite previous research that reported positive results after vitamin D supplementation. (Linde et al. 1972). The drug is reducing the amount of vitamin D available for hydroxylation; therefore supplemented vitamin D would be expected to counteract these effects to an extent. The effects of vitamin D on the body include calcium absorption in the intestines, cell transcription and gene regulation in the parathyroid gland, phosphate transport and absorption, and numerous actions on bone. In bone, vitamin D regulates the transcription of two bone matrix proteins, type I collagen, and osteocalcin. The synthesis of type 1 collagen is repressed while the synthesis of osteocalcin is induced (Bringhurst et al. 2003).

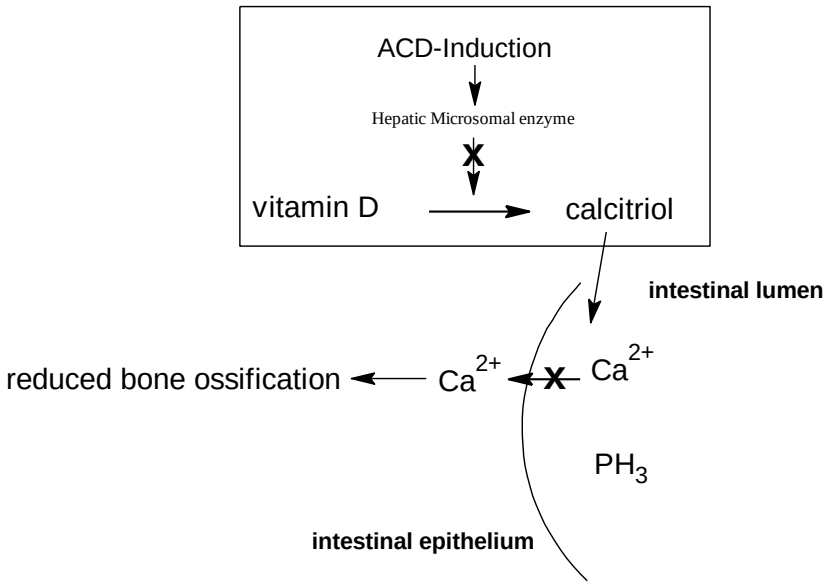

Figure 2: The induction of the hepatic microsomal enzyme caused by anticonvulsant drugs resulting in decreased calcium and phosphorous absorption and reduced bone ossification.

The anti-epileptic drugs carbamazepine and valproic acid are the most commonly used drugs in epileptic patients, with approximately two-thirds of such patients taking these drugs (Ray et al. 2002). These drugs have not only been found to cause reduced bone density and higher incidences of bone disease, but are also implicated in spina bifida and other neural tube embryonic defects (Ray et al. 2002). In mouse and human embryos, valproic acid has been found to induce a number of neural tube defects (Kultima et al. 2004). Carbamazepine has also been found to cause spina bifida in 1% of cases when used by pregnant women (Oakshott and Hunt 1991). Congenital abnormalities are found more often in newborns whose mothers have been on mono or polytherapy anticonvulsant drugs. Czeizel et al. (1992) found that carbamazepine caused the second highest number of cases of congenital defects including spina bifida, cardio

vascular problems, and cleft palate. The teratogenic effects of these drugs in combination with their effects on bone density are interest because the mechanisms in which they affect bone may be used to affect developing bone. The studies in the literature up to this date have only reviewed the effects of anticonvulsant drug therapy on mature bone, and the effects on embryonic bone development are unknown.

Bone Development

The basic process of bone development is conserved among vertebrate organisms. During embryonic development, the skeleton forms as a cartilage frame produced by cells called fibroblasts. This cartilaginous frame is replaced by bone to add rigidity and strength. Bone is formed around (in perichondral bone) and within (in endochondral bone) the cartilage of the embryonic endoskeleton. When bone begins to develop, the fibroblasts change function to form osteoblasts then form a matrix of collagen and protein polysaccharides called as osteiod. This matrix is then calcified by the binding of calcium phosphate (hydroxyapatite) to the collagen fibers. As seen in Figure 3, the bone forming cells become trapped forming spaces called lacunae within the osteiod matrix. As bone develops, the randomly organized collagen bundles are moved into parallel sheets (lamellae) which allows for different directions of fiber growth, and therefore strength (Walker & Liem 1994).

Ossification of the skeleton occurs as the bone and cartilage continue to grow. Ossification occurs first around the middle of a long bone in the formation of a perichondral bone collar called the diaphysis. As this newly formed bone ossifies, it becomes the periosteum and calcium is deposited throughout the matrix. As the cartilage breaks down to leave only bone, the matrix becomes honeycombed, vascularized, and

then filled with bone marrow, illustrated in Figure 4. This ossification continues down the length of the bone until it reaches the cartilaginous epiphysial plate, which does not ossify, but continues to grow throughout adult life.

Bone ossification is a continual process during the life of a vertebrate. Perichondral bone continues to be added around the periphery of the primordial cartilage skeleton increasing the girth of the bone shaft. During this formation, the bone is constantly being remodeled in order to allow for expansion and to meet mineral needs of the body's metabolism. Cells called osteoclasts secrete acid in the bone that breaks it down and forms Haversian canals. Osteons are the marrow containing canals that make up the Haversian system. With this system, most of the bone is found on the periphery as a dense tissue called compact bone. Depending of the load of bones, the calcification can occurs in varying degrees. Load bearing bones tend to have a higher mineral content (67%) (Walker & Liem 1994). This process depends on regular levels of calcium, phosphate, and alkaline phosphatase, which may all be altered by an irregularity of vitamin D (Raisz et al. 2003). In amphibians, ossification of limb bones normally occurs in the mid-larval stages and is marked by an increase in serum calcium levels (Feder &Burggren 1992).

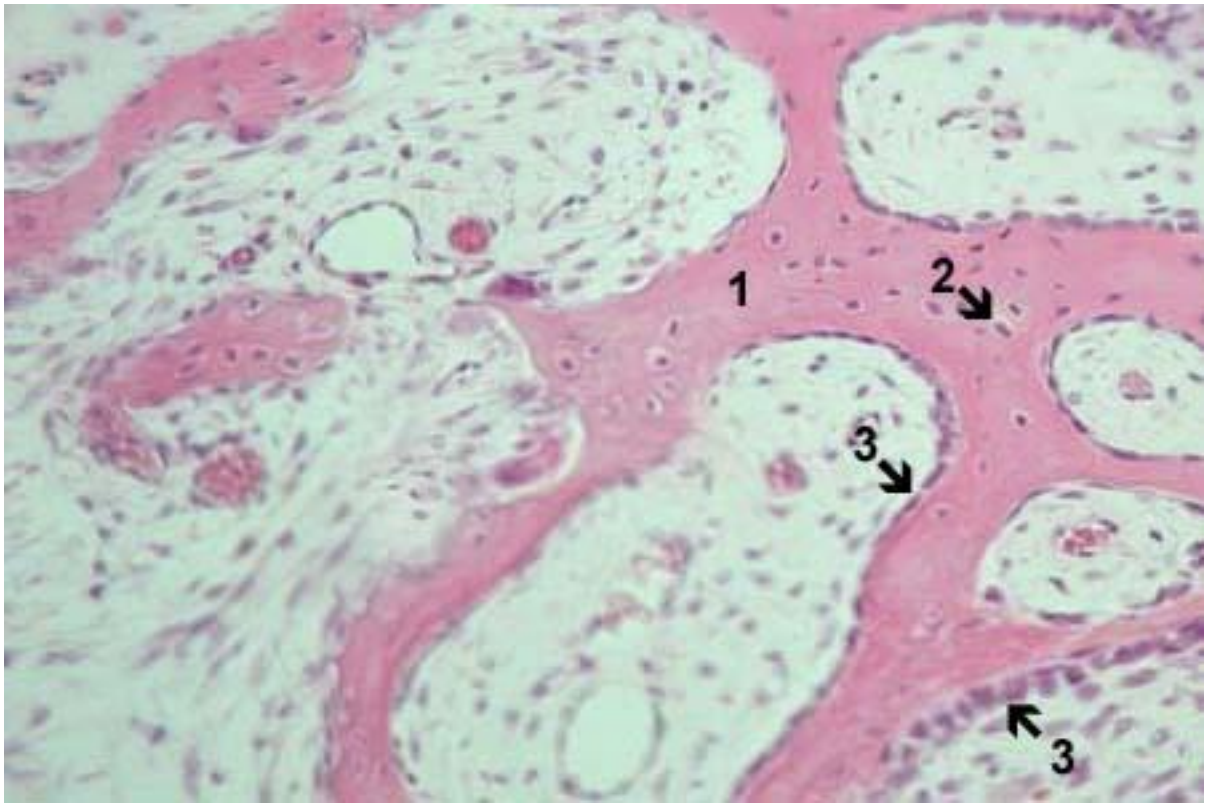

Figure 3: Developing bone illustrating the intercellular bone matrix (1), osteocytes (2), and the periosteum (3). Image from <http://www.histol.chuvashia.com/atlas-en/connective-01-en.htm>

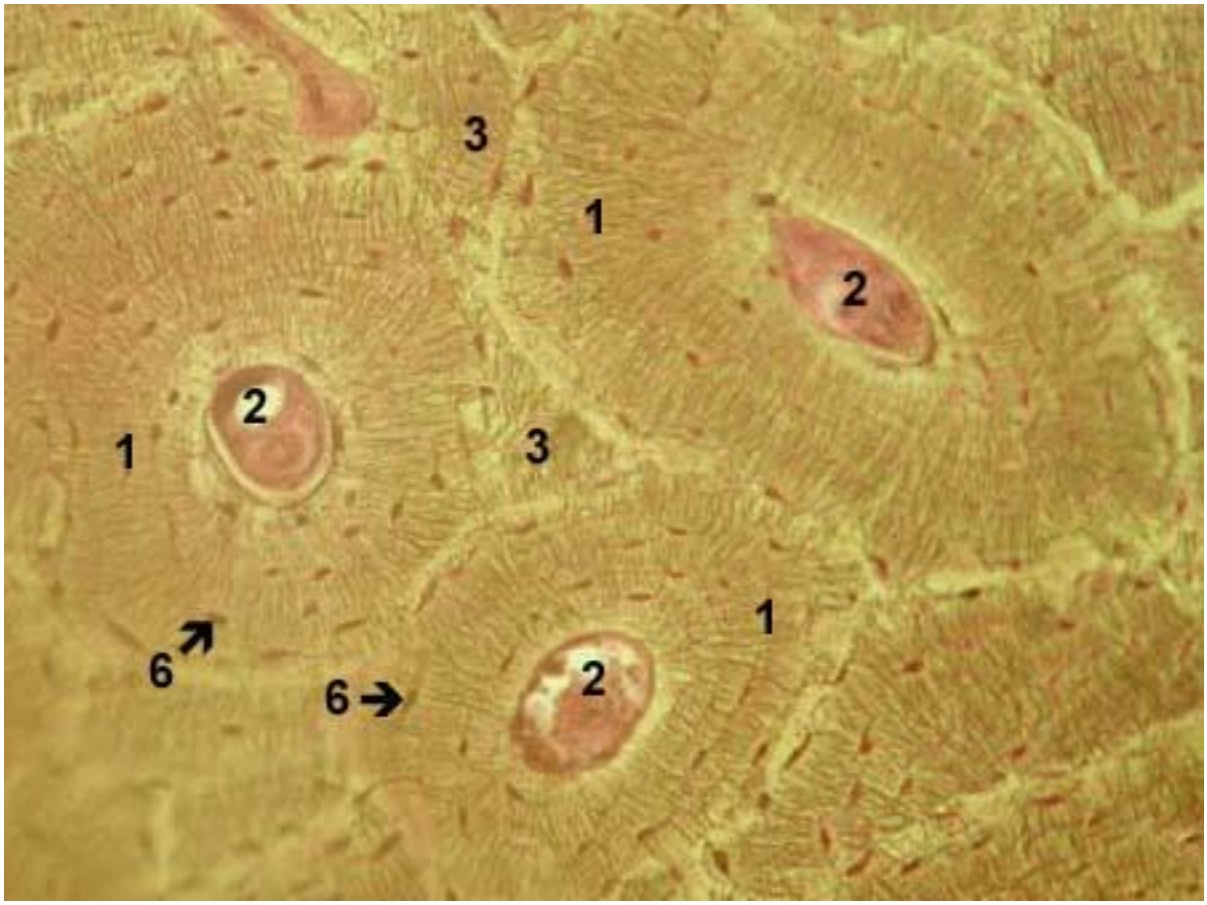

Figure 4: Image of mature bone depicting the haversian system (1), haversian canal (2), interstitial bone (3), and osteocytes (6). Image from <http://www.histol.chuvashia.com/atlas-en/connective-01-en.htm>

With a few exceptions, anuran bone development occurs just as in other vertebrate organisms. One subtle difference is that amphibians have reduced number of trabaculae and reduced quality of the epiphyseal growth plates. Also endochondral ossification is a delayed process (Miura et al 2008). The sequence of ossification of the skeleton of metamorphosising *Rana pipiens* tadpoles has been determined and related to the Taylor and Kollros developmental stages. Initially, ossification begins at the

parasphenoid bone during the stages IV-IX. As development progresses, the ossification process moves along the vertebral column. The limbs ossify starting from the inside and progressing outwards (Kemp & Hoyt 1969). The sequence of tadpole ossification can be seen in Table 1. This sequence of ossification is not altered by the addition of thyroxin to the tadpoles except in the skull bones.

Table 1: Sequence of bone ossification of *Rana pipiens* tadpoles related to Taylor and Kollros stages (Kemp & Hoyt 1969.)

Order of ossification of bones of appendicular and postcranial axial skeleton in Rana pipiens tadpoles as indicated by alizarin staining

Bones	Taylor-Kollros stages and numbers examined																		
	IV (18)	V (14)	VI (13)	VII (14)	VIII (11)	IX (10)	X (14)	XI (11)	XII (10)	XIII (13)	XIV (10)	XV (10)	XVI (10)	. . .	XX (14)	XXI (19)	XXII (12)	XXIII (9)	XXIV (14)	XXV (6)
	Percentages of specimens ossifying																		
Hindlimb																			
Femur						0	57	55	100										
Tibiofibula						0	57	55	100										
Tarsals						0	14	55	100										
Metatarsals						0	7	18	80	100									
Phalanges									0	23	70	100							
Pelvic girdle																			
Ilium						0	14	55	100										
Ischium															14	58	75	100	
Pubis (calcified cartilage)																		0	7 50
Forelimb																			
Humerus						0	7	55	100										
Radioulna							0	45	90	100									
Carpals																			0 17
Metacarpals									0	62	90	90	100						
Phalanges										0	50	90	100						
Pectoral girdle																			
Scapula								0	20	77	100								
Coracoid								0	20	62	100								
Clavicle									0	15	50	100							
Suprascapula									0	8	50	100							
Sternum																			
Mesosternum																		0	7 100
Omosternum																			0 50
Vertebral column																			
Vertebrae	0	7	8	29	9	100													
Urostyle					0	10	43	27	60	92	100								

Carbamazepine as a test drug

This study will use carbamazepine to test the effects of anticonvulsants on bone formation in developing frog bone. Carbamazepine is used for its anticonvulsant properties in epileptic patients. This drug is an inhibitor of voltage gated sodium channels, which allow the initiation and sustainability of movement. Carbamazepine (5-H-dibenz[b,f]azepine-5-carbozamide) is one of the more widely used anti-epileptic drugs on the market (Mantegazza et. al. 2010) along with phenobarbital, phenytoin, and valproic acid (WHO 2005). In 2005 93.1% of countries listed carbamazepine as an essential drug to their country's health (WHO 2005). The wide use of carbamazepine has led to its presence in many water supplies, and it has become a marker of anthropogenic influences in waterways (Clara et. al 2003). An ELISA measuring the presence of the drug in surface waters of Berlin found it to be present in every water sample in concentrations of 0.05-3.2 μg/L(Bahlmann et. al. 2009) . It is not surprising to find carbamazepine in waterways because it is extremely stable in aquatic environments due to its insolubility in water. It can be measured in waters recently treated at sewage treatment plants because it is not absorbed or degraded during the treatment process. Table 2 includes environmental levels of carbamazepine.

Table 2: Environmental levels of carbamazepine in water sources found in the last decade.

Environmental Level of Carbamazepine	Comments	Source
0.05-3.2 μg/L	German surface water	Bahlmann et al 2009
3.8 μg/L	Influent wastewater treatment plants	Heberer 2002
0.5 μg/L	German surface water	Zhang et al 2008
0.45 μg/L	German surface water	Liebig M, Moltmann JF, Knacker T (2006) Environ Sci Pollut Res 13:110–119
900 ng/L	German groundwater	Sacher et al 2001

The purpose of this study is to investigate the effects of the anti-convulsant drug Carbamazepine on development of the bone during the metamorphosis of *Rana sylvatica* tadpoles. Previous studies have examined the effect carbamazepine has on developed and remodeling bone. The metamorphosis stages of tadpole development allow investigation of the bone development during carbamazepine exposure. Wood frog tadpoles were chosen as subjects for this study because much is known about skeletal development in amphibians, they are easy to maintain, and their integumentary system allows water-borne substances to pass into circulation. It is hypothesized that carbamazepine exposure will reduce bone calcification and development by inhibiting absorption of calcium and phosphorous into the blood stream.

CHAPTER II
Materials and Methods

Animals

This study utilized 16 *Rana sylvatica* tadpoles obtained from a single clutch collected by Dr. Crain for Biology 414: Developmental Biology. Animal husbandry and use was approved by IACUC 201004 (see Appendix 1). Each tadpole was placed alone in a 250 mL beaker of 150 mL fresh water. Tadpoles were grouped according to developmental stage as follows: 5 in Early Treated Group (Gosner stage 29), 5 in Late Treated Group (Gosner stage 35), 3 in Early Control Group (Gosner stage 29), and 3 in Late Control Group (Gosner stage 35). These two stages were chosen for the experiment because at stage 29, the tadpoles have small hindlimb buds, while in stage 35, the hindlimbs are almost fully developed (Gosner 1960). Fresh water was prepared using Start Right water purifier and left to sit for at least 12 hours. The tadpoles were allowed to acclimate in their beakers for one day prior to exposure.

Exposures

After the period of acclimation, 20μL of a solution of 3mg/mL Carbamazpine (CBZ; Sigma Aldrich C4024) in 95% ethanol was added to each of the individual tadpole tanks in both early and late treated groups (the final concentration of of CBZ in each tank was 0.4 μg/mL). To both early and late control groups, 20 μL of 95% ethanol was

added. The tadpoles were dosed considering the human therapeutic range of CBZ at 50mg/kg. Assuming that each tadpole is only exposed to half of the water in his tank every day due to the observed motility rate, the dosage for 150 mL of water is twice that of the normal therapeutic human dose. Every five days, half of the water in each tank (75mL) was removed and replaced with fresh water. Each tank was then dosed again with 10 μL of the 3mg/ml CBZ solution to maintain 0.4 μg/ml.

Euthanasia and Fixation for Histology

The tadpoles were removed from their tanks and euthanized using 400 mg/L solution of MS 222 when they attained developmental stage 42 -44 (Gosner). The tadpoles were collected during these stages in development because the forelimbs were completely developed by this time and in the next few stages, the tadpoles lose the function of their gills and can no longer be treated. This resulted in 3 weeks of exposure for the early dosed group and 2 weeks of exposure for the late group. Each tadpole's left forelimb was removed, and the forelimb and body were placed in Bouin's fixative (see Table 2).

Histology was performed on the left humerus, which was selected for bone density determination because it is one of the first bones to ossify in the development of bone during metamorphosis (see Table 1). This bone is also relatively small and would not need to be decalcified in order to prepare histology slides. The left forelimb was chosen because the right forelimb of several tadpoles did not emerge. The forelimbs remained in the Bouin's fixative for 2-3 weeks before being cleared of the fixative in 70% ethanol. This process was repeated for three more weeks to ensure the removal of excess Bouin's fixative.

Table 3**:** Reagents used in the preparation of Bouin's fixative used for preserving the dissected *Rana sylvatica* limbs.

Component	Volume Used (mL)
Picric acid (saturated aqueous)	75
Formaldehyde	25
Glacial acetic acid	5

Sectioning and Staining

Next the tissue was dehydrated by containing the tissue in a plastic cassette and putting it in 80% ethanol for 2 hours, then 95% for 1.5 hours, 100% for 1 hour, and a second 100% for one hour. The tissue was cleared in CitriSolv for 1 hour. The forelimbs were embedded individually in wax blocks by soaking in four series of paraffin waxes each for an hour under increasing pressures (12, 15, 21, then 25 psi). The tissue was carefully placed in a mold filled with paraffin wax. This was allowed to set. Once the block hardened, it was trimmed and then sectioned on the microtome at 12 micrometers. The ribbons of sections were mounted on slides and then stained using Hematoxylin and Eosin staining technique (Presnell and Schreibman 1997). This process is detailed in Table 3. The slides were loaded into racks in order to be moved from solution to solution. In the steps that require running water, the rack was placed under a gentle current of water for the appropriate amount of time. After staining, permount was used to add the cover slip to the slides. The slides were allowed to dry on a level surface overnight before analysis.

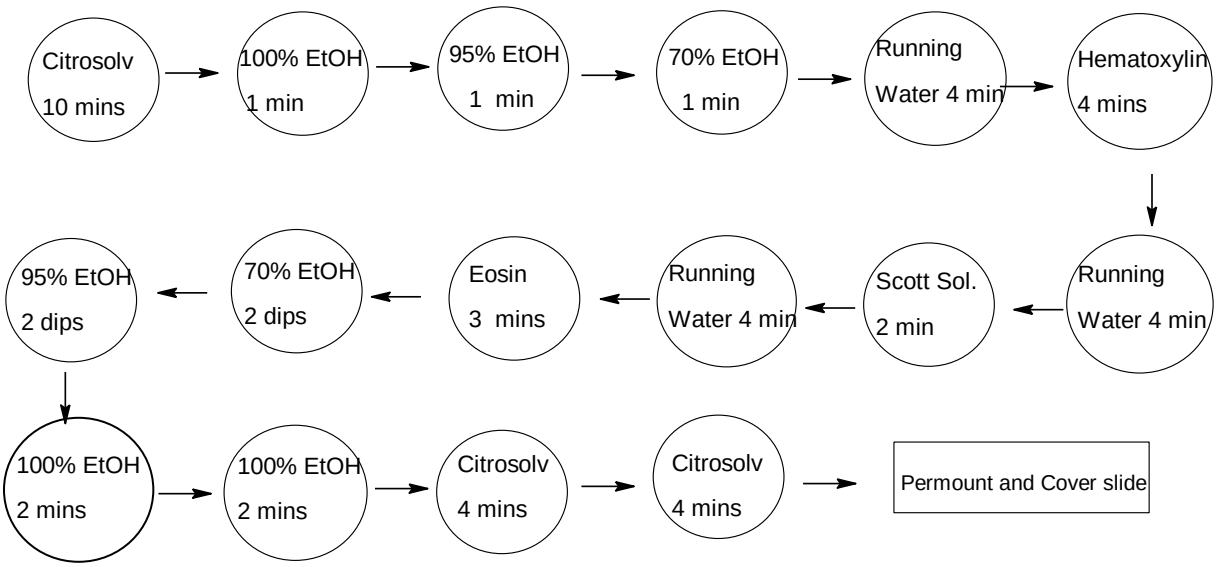

Figure 5: Hematoxylin and eosin staining protocol (Presnell and Schreibman 1997) used to stain the slides for histological analysis.

Morphometrics

The length of the preserved right humerus of each frog was measured in millimeters by dissecting the bone from the body and measuring from head of bone to end under a dissection microscope.

The slides of the left humerus were analyzed at 100X on a section midway down the shaft of the humerus for each animal. A stage micrometer was used to calibrate the ocular micrometer. For each bone shaft, five consecutive sections were measured in millimeters for humerus diameter and diameter of remaining cartilage. In each of these sections the amount of ossified bone was determined by determining the area of both the entire shaft, and the remaining cartilage core and subtracting the area of the cartilage core from the area of the entire shaft as shown in Figure 6. These measurements were used to determine percent area of ossification in the field of view for each animal.

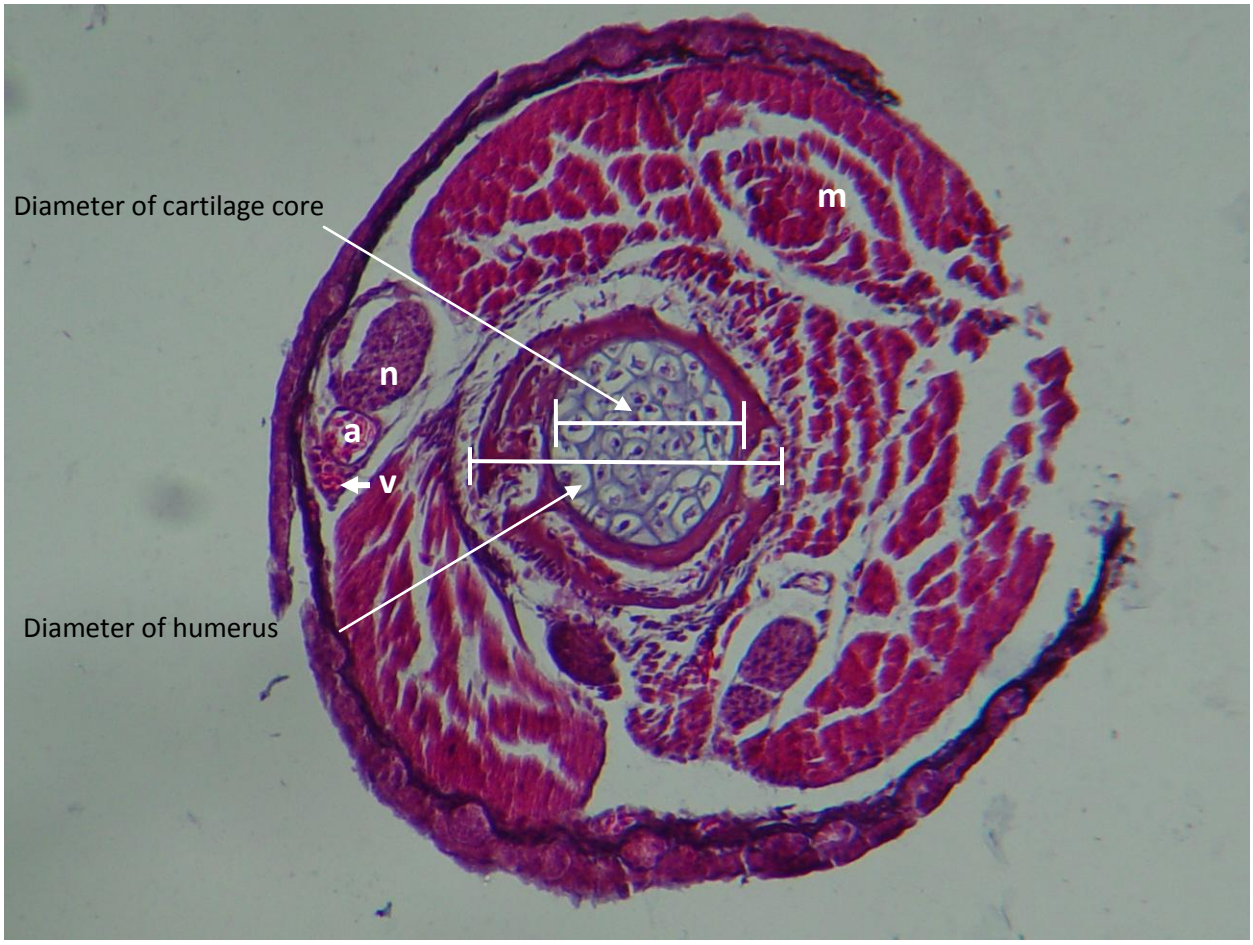

Figure 6: A cross section of frog forelimb at 100X indicating method for measuring the diameter of the cartilage core and the entire humerus diameter. The area around the cartilage is ossified bone and developing marrow. Muscle (m) is present around the humerus shaft as well as nerves (n), veins (v) and arteries (a).

Statistical Analysis

For each humerus the measurements (n=5 for length, diameter, and ossification) were averaged for statistical analysis. A t-test was performed comparing the control and carbamazepine exposed groups of both the early and late groups to analyze statistical significance (Microsoft Excel alpha=0.05).

CHAPTER III
Results

Carbamazepine exposure did not have any significant effects on the bone development of *Rana sylvatica* tadpoles during metamorphosis. No statistical difference is present in comparison of the CBZ exposed groups and the control groups of both the early-dosed and the late-dosed groups in humerus length (early p=0.497, late p=0.953), diameter (early p=0.420, late p=0.345), or percent ossification (early p=0.757, late p=0.337) of the humerus. Figure 7 and 8 show examples of humerus cross-sections in each group, early control, early CBZ, late control, and late CBZ.

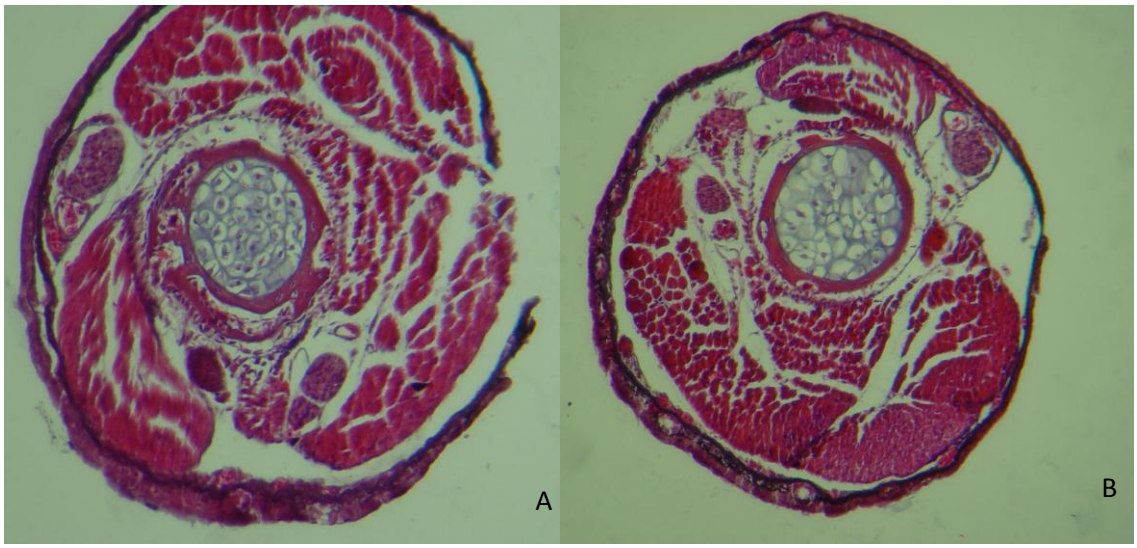

Figure 7: Images of the humerus cross –section at mid-shaft of the early control (A) and the early CBZ exposed group (B) at 100X. Note that in both control and CBZ exposed the humerus is made up largely of cartilage. The control humerus appears to have a larger percent ossification around cartilage core, however this was not the trend in all.

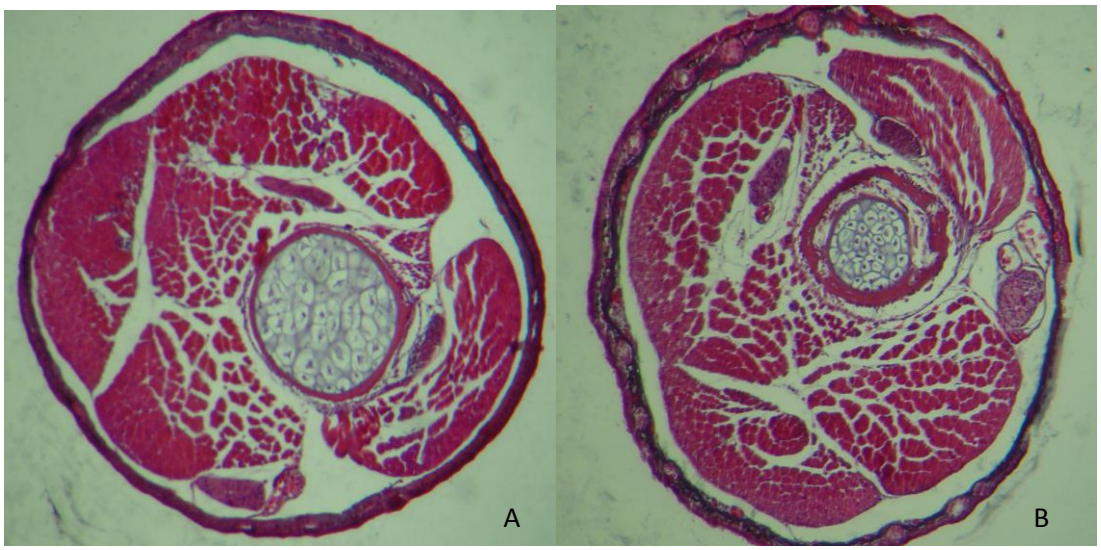

Figure 8: Images of the humerus cross –section at mid-shaft of the late control (A) and the late CBZ exposed group (B) at 100X.

In both the early and late groups, CBZ did not significantly affect the length of the humerus in comparison to the un-exposed control groups ($p>0.05$). Figure 9 compares the mean humerus length of each group plus and minus one standard error. The mean diameter of the humerus in both the early and late groups showed no significant difference between the control groups and the CBZ controled groups ($p<0.05$). Figure 10 compares the mean diameter (mm) with one standard error in each of the groups. Percent ossification of the tadpole humerus at the area where measurements were taken was also not affected by exposure to carbamazepine. A t-test between the control and carbamazepine exposed in both the early and late groups returned a p value less than 0.05. Figure 11 compares the mean percent ossification between the early dosed (CBZ and control) and the late dosed (CBZ and control) groups with one standard error.

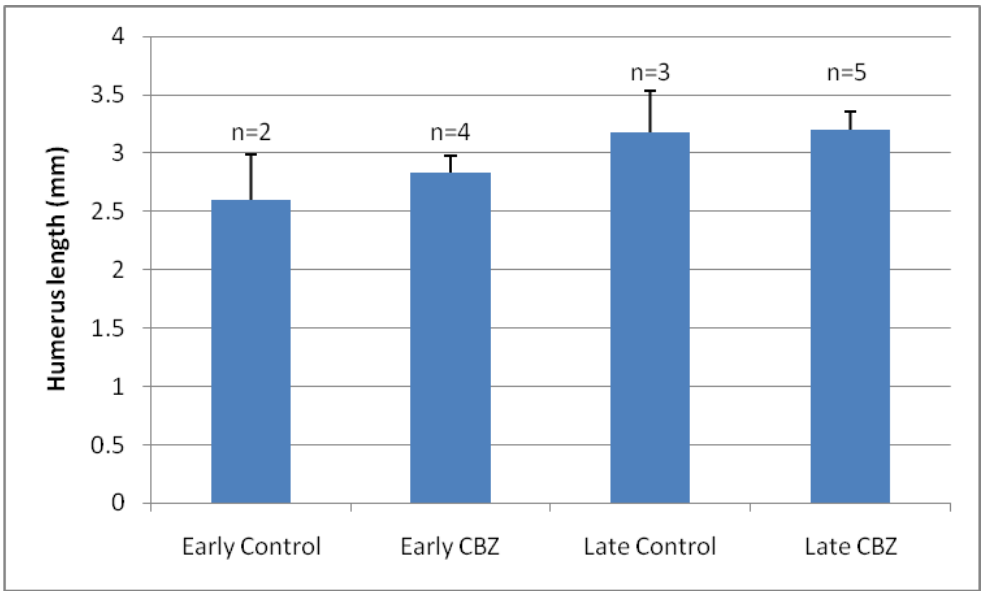

Figure 9: A comparison of mean humerus length in milimeters (± 1SE) of the early dosed (between CBZ exposed and control) and the late dosed (between CBZ exposed and control) groups of tadpoles.

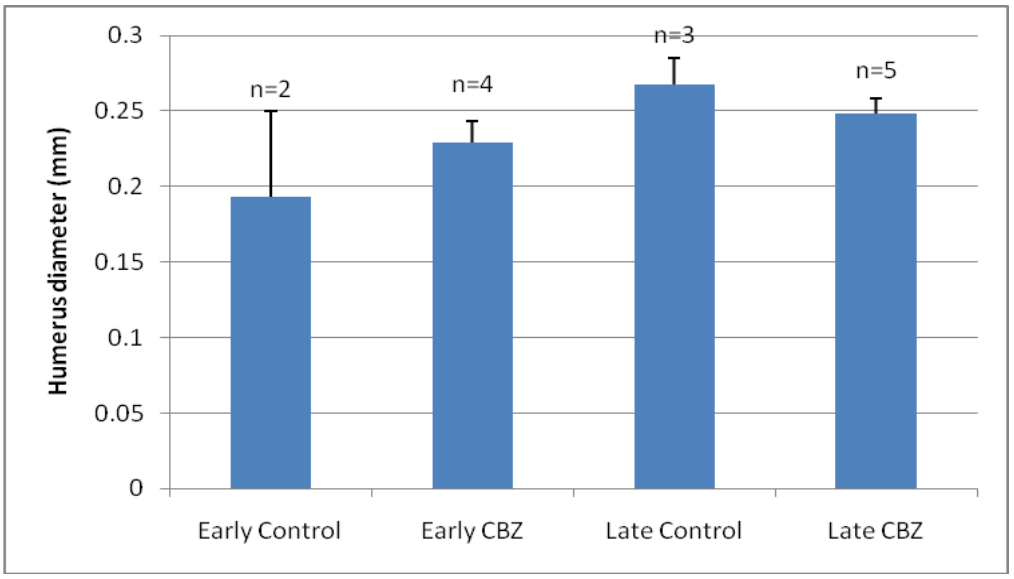

Figure 10: A comparison of mean humerus diameters in milimeters (± 1 SE) of the early dosed (between CBZ exposed and control) and the late dosed (between CBZ exposed and control) groups of tadpoles.

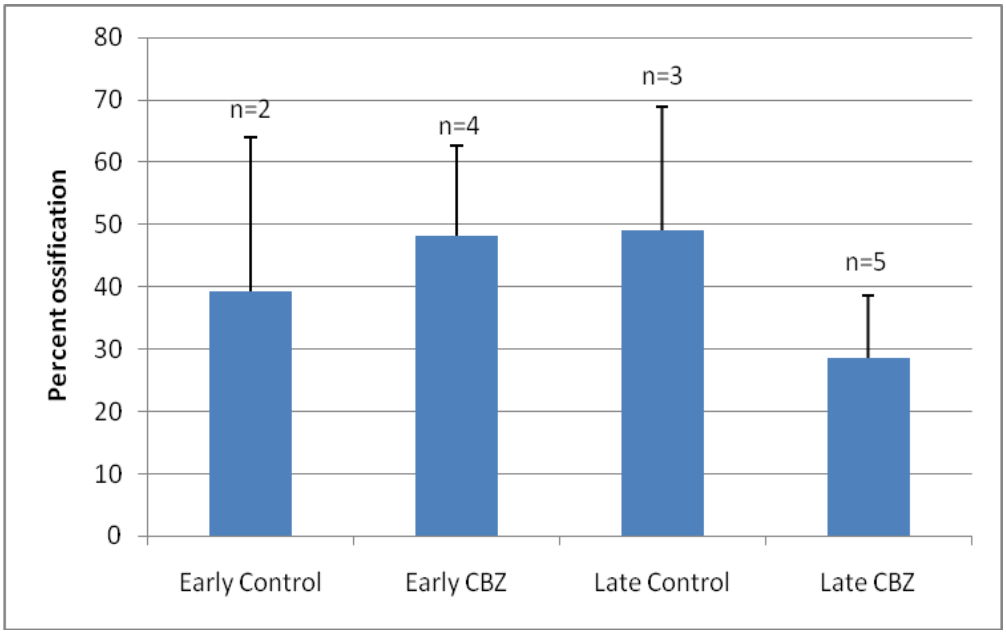

Figure 11: Mean percent ossification of the humerus shaft (±1 SE) comparing CBZ exposed and control of the early-dosed and the late-dosed groups of tadpoles.

Since the CBZ groups were not significantly different than the control groups, they were combined to compare bone development early dosed and late dosed groups for each measurement taken. The t-test performed comparing early and late groups of length, diamter, and percent ossification yieled no statitical significance ($p=0.060$, 0.065, and 0.593 respectively). Figure 12 is a comparison of the mean humerus length in milimeters between the early-dosed and late-dosed group with one standard error. The difference in mean length between the early and late groups was not significant ($p>0.05$). The humerus diameter (mm) between the early and late groups is compared in Figure 13 with one standard error. Figure 14 compares percent ossification between the early and late groups. Though the difference between the early and late groups for these measurements is not significant, the differences are apparent.

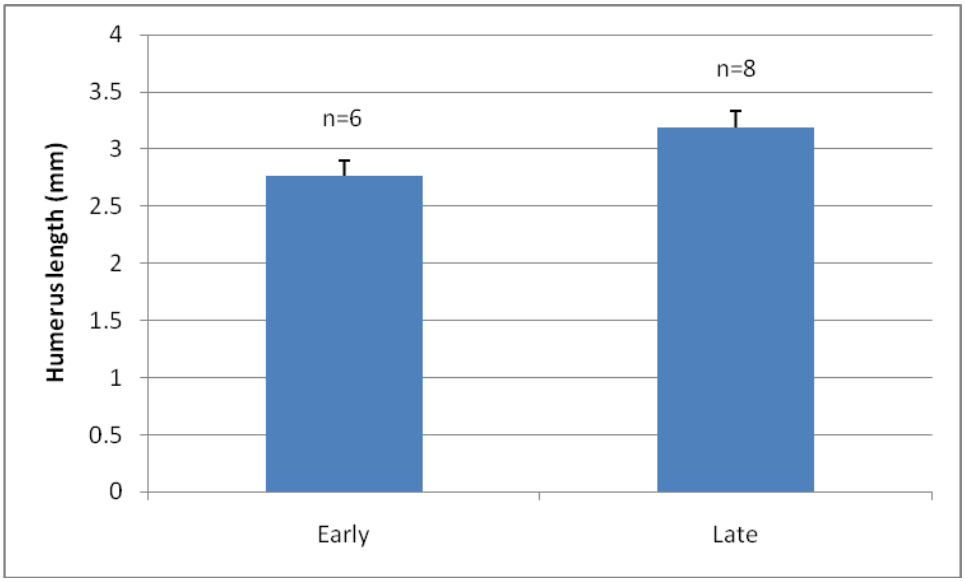

Figure 12: A comparison of the mean humerus length in milimeters (± 1SE) between the early and late groups (CBZ exposed and control combined).

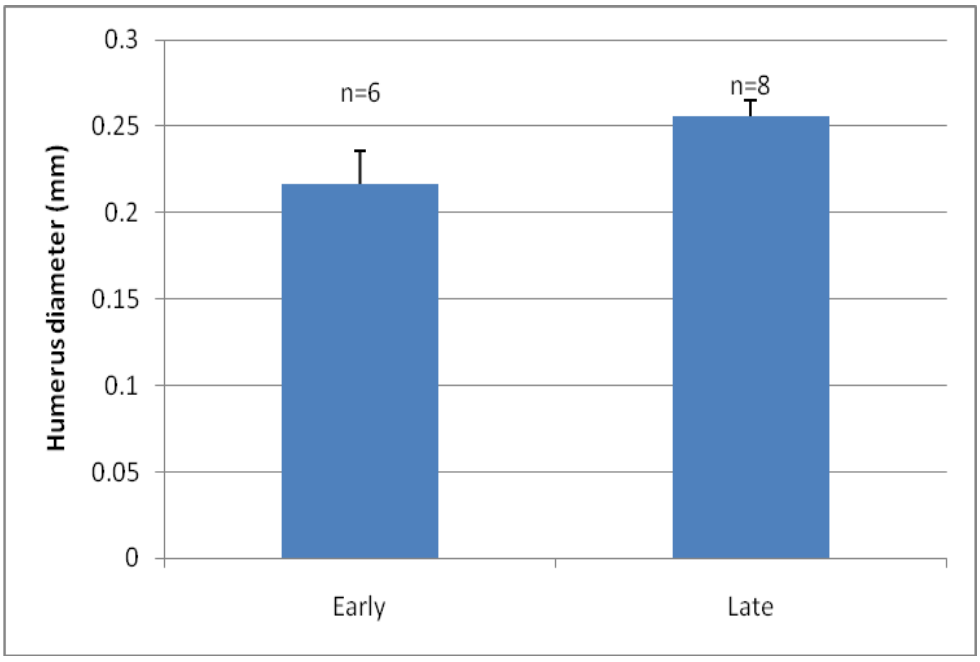

Figure 13: Mean humerus diameter in milimeters (± 1 SE) compared between the early and late groups (CBZ exposed and control combined).

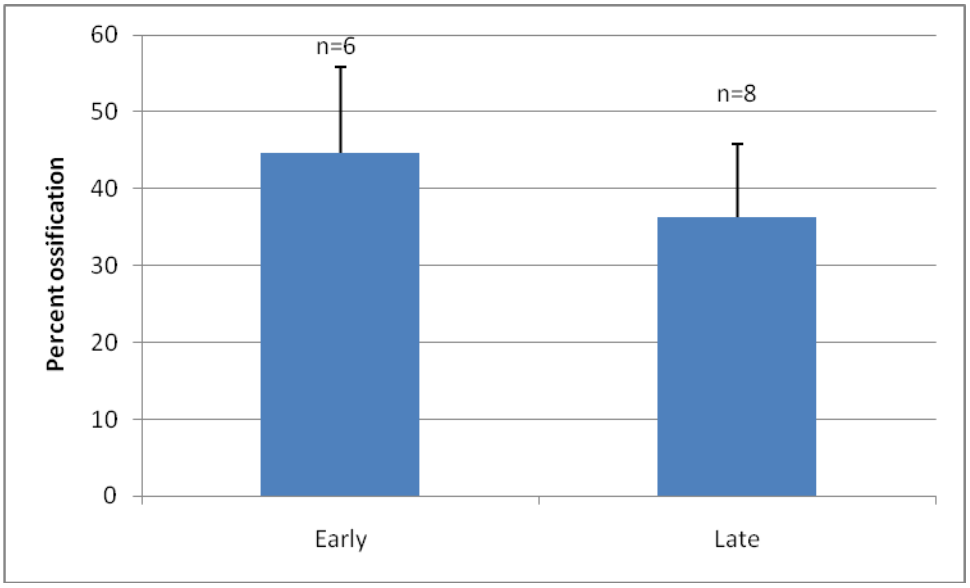

Figure 14: A comparison of percent ossification (± 1 SE) between the early and late groups (CBZ exposed and control combined).

CHAPTER IV
Discussion

Bone development of *Rana sylvatica* tadpoles was not significantly affected after exposure to carbamazepine during metamorphosis. When osteogenesis was quantified through measurement of humerus length, diameter, and percent ossification, neither the early or the late-dosed groups were significantly affected by carbamazepine exposure. The CBZ exposed tadpoles of the late group however did have an apparent (although not significant) decrease in humerus diameter and ossification, while the CBZ-exposed in early group showed an apparent increase in these quantities. Comparison between early and late groups for each quantification method (humerus length, diameter, and percent ossification) also showed no difference between groups but the humerus length and diameter did appear to be increased in the late groups. Overall, carbamazepine did not affect bone development of exposed tadpoles. These results do not support the hypothesis.

Other studies investigating the affects of carbamazepine on bone only present results regarding remodeling and disease in adult bone. In general anti-epileptic drugs cause hypocalcemia, hypophosphatemia, reduced serum levels of biologically active vitamin-D metabolites, and elevated serum parathyroid hormone (LaRoche & Helmers 2004, but the mechanism of decreased bone mineral density in adults differs depending on the drug administered. Drugs in the same class as carbamazepine (phenobarbital,

phenytoin, primidone) cause induction of the hepatic cytochrome P450 enzyme, CYP3A4. Induction of the cytochrome P450 system leads to metabolism of calcitriol into in-active metabolites, which in turn leads to decreased bone mineralization, decreased serum calcium, and decreased bone-density (Pack 2003). The reduced serum calcium stimulates a positive feedback from the parathyroid glands, up-regulating the amount of parathyroid-hormone secreted (secondary hyperparathyroidism).

As mentioned before, there are different mechanisms, some unknown, by which antiepileptic drugs affect bone mineral density and development. Induction of cytochrome P450 enzymes is the mechanism of action found of carbamazepine, phenobarbital, phenytoin, and primidone (Minzter 2006). Another heavily prescribed medication for epilepsy is valproic acid or valproate. Studies regarding the effect of valproic acid on bone health have returned mixed results. Early studies found no significant affects on bone health(Gough et al 1986 and Davie et al 1983), while a later study found increased serum calcium and lowered calcitriol (Sato et al 2001). The mechanism by which Valproic acid, an inhibitor of the cytochrome P450 enzymes, reduces bone mineral density is largely unknown. It has been postulated that the drug has direct effects on bone cells causing reduced calcium absorption, hyperparathyroidism, and calcitonin deficiency (Pack 2003).

Parathyroid hormone increases bone breakdown and resorption in adult bone. The new bone created during bone remodeling is not mineralized as effectively because of low serum mineral levels. During fetal skeleton development, parathyroid hormone does not have a significant role. Instead, parathyroid hormone related-peptide (PTHrP) provides a critical function in fetal endochondral bone formation. PTHrP is unlike PTH

because it is secreted by many tissues throughout the body. Studies suggest it acts as a calciotropic hormone during fetal life, stimulating transport of calcium across the placenta. The role of PTHrP in fetal bone development becomes clear when severe skeletal abnormalities occur in PTHrP delete mice. (Bringhust et. al 2003). During fetal bone development, PTHrP regulates differentiating chondrocytes. Overexpression of PTHrP causes a delay in chondrocyte differentiation and therefore chondrocyte mineralization. The pattern of chondrocyte mineralization is dependent on the differentiation of chondrocytes (Chung et al 1998). In amphibian models, the parathyroid is developed during metamorphosis but this is not the only gland responsible for calcium regulation. Amphibians retain some control of calcium levels through the pituitary gland and the paraphysis (Bergwitz et al 1997). The parapysis cerebri is a gland located in the third ventricle of ancestral vertebrates (amphibians and reptiles) that plays a role in calcium metabolism (Hinton et al 1990). Therefore, the ossification of bone in the current study was not only under control of the parathyroid. The effects of antiepileptic drugs on the endocrine systems of amphibians have not been studied and therefore the reaction of the pituitary and paraphysis gland to carbamazepine is not known.

In mammals, the pituitary gland does not regulate serum mineralization levels (Guyton & Hall 2006), therefore any effects of hyperparathyroidism on bone would be exaggerated. Ancestral vertebrates do not have a parathyroid gland, but proteins resembling PTH and PTHrP are still produced and retain a function in mineral homeostasis (Gensure & Juppner 2005). Amino acid identity of PTHrP is significantly conserved in all vertebrates (Ingleton 2001). The amphibian parathyroid gland evolved recently, which is why calcium levels are still controlled by other areas. Antiepileptic

drug use is implicated in the development of secondary hyperparathyroidism, but it is unknown whether this condition may affect the production of PTHrP (Miao et al 2001). In the case that hyperparathyroidism causes and increased level of PTHrP, chondrocyte differentiation would be delayed and ossification would occur in an irregular pattern. The current study may not have found any results because the pituitary glands in *Rana sylvatica* regulated calcium levels in place of PTHrP. The literature lacks studies regarding the effects of anti-epileptic drugs on the parathyroid gland and the action of PTHrP on ossification of endochondral bone. This would be an interesting direction to investigate in the examination of anti-epileptic drug related bone disease. Studies regarding the affects of antiepileptic drug use on fetal development, which is what this study modeled, are restricted to neural tube and other congenital defects.

In summary, the current study found evidence that carbamazepine does not affect developing bone in an amphibian model. In amphibians, the calcium levels during osteogenesis are regulated by a recently evolved parathyroid gland, and by the conserved control through pituitary and paraphysis. As the effects of carbamazepine on osteogenesis likely rely on the drugs ability to alter the parathyroid axis, it is unknown if human osteogenesis would be altered by carbamazepine exposures. Conducting this study on an animal that regulates calcium in the same manner that humans do would eliminate the possibility of calcium regulation by another route than the parathyroid. In this study, the amphibian was not an appropriate model. The long term side effects regarding bone are restricted to already developed bone and the remodeling taking place within bone. This study investigated the effects of the anti-epileptic drug, carbamazepine, on developing bone through the possible induction of the hepatic

cytochrome enzyme system and found no significant difference in bone health. It is important, however to continue exploring the consequences of anti-epileptic drugs such as carbamazepine on developing bone adjusting dosage and duration of exposure.

Appendix A: IACUC

MARYVILLE COLLEGE
Institutional Animal Care & Use Committee (IACUC)
Student Animal Research Form

Provide information after each bold item
Student Name: Hillary Brown

Student Email Address: Hillary.Brown@mv.maryvillecollege.edu

Date:2/24/2010

Senior Study Advisor: Dr. Crain

Species to be used: ***Rana sylvatica***

Age of animals: tadpole stages

Number of animals in study: 45

Duration of study: April-August 2010

Location of animals during the study (building and room) : Sutton Science Center

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Hillary Brown	566-8359	566-8359	566-8359
Dr. Crain	865-981-8238	379-1706	850-5703

Husbandry Requirements: Is anything other than routine care and equipment required?
YES NO If "YES", please list below.

What will happen to the animals at the end of the study? If euthanasia is required, state the methods.
The animals will be euthanized using MS222 0.5%

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

Maryville College IACUC Approval Number: 20100 4
Date Approved: 3/1/10
Signed:

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