EFFECTS OF THE ANTIBIOTIC ENROFLOXACIN ON THE GASTROINTESTINAL TRACT IN METAMORPHOSING *XENOPUS LAEVIS* TADPOLES

A Report of a Senior Study

by

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ABSTRACT

There are many sources through which human-used antibiotics are being released into the environment, including sewage treatment facilities and livestock farms. Environmental antibiotics have potential ecological effects on amphibians, especially herbivores due to the role of symbiotic bacteria in allowing herbivory. The objective of this study was to investigate the effects of the antibiotic enrofloxacin on the intestinal flora and morphology of the gastrointestinal tract of herbivorous tadpoles. *Xenopus laevis* tadpoles, ranging from NF stage 52 to 65, were exposed to enrofloxacin (Baytril), intestines were removed, and fecal matter was extracted. Mean numbers of fecal bacterial colonies were not different at the 1:100 dilution ($p=0.879$) or the 1:10,000 dilution ($p=0.690$), but the types of bacteria from control tadpoles appeared different from exposed tadpoles based on colony morphology. Significant differences in intestinal epithelial cell height were found between doses ($p=0.014$), between stage ($p=0.010$), and with interaction between dose and stage ($p=0.035$), whereas no differences were found in epithelial cell density. Enrofloxacin was shown to decrease intestinal epithelial cell height and alter intestinal flora in *X. laevis* tadpoles. Because antibiotics can change both GI tract epithelial structure and microflora, environmental concentrations have the possibility of altering amphibian populations.

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CHAPTER I

INTRODUCTION

Water Pollutants

There have been increasing concerns in recent decades over the possible adverse human and ecological health effects resulting from the production, use, and disposal of agricultural, urban, and industrial chemical pollutants (Daughton and Ternes 1999). In the U.S., water-borne environmental contaminants can be categorized based on the source from which they entered the waterways: surface water and groundwater. Surface water pollutants are from both point (e.g. pipes, ditches, sewage treatment plants, factories, and city storm drains) and nonpoint sources. Polluted runoff is caused by rainfall or snowmelt that picks up and carries natural and human-made pollutants and deposits them into watersheds as it moves over the ground (U.S. EPA). An example of contamination from a non-point source is nitrogen compounds from fertilized agricultural lands. As for the other major category of sources of contaminants, groundwater sources are spills or releases of chemical or radionuclide contaminants into the soil.

There are many different specific contaminants found in waterways in the U.S. This long list includes heavy metals and other trace elements, radionuclides, medications and personal care products, pesticides and herbicides, nutrients such as nitrogen and

phosphorous, viruses and other pathogens, and petroleum products and related additives. Some of these contaminants are more problematic than others. The U.S Environmental Protection Agency (EPA) published its first list of unregulated contaminants of concern in 1988, known as the Contaminant Candidate List (CCL). Initially, the list contained 400 contaminants but was limited to 50 chemical and 10 microbial contaminants to help the EPA prioritize its research (Christen 2002). Published in 2009, CCL 3 is the most current version of the list containing unregulated contaminants that are known or anticipated to occur in public water systems. The CCL 3 includes 104 chemicals and 12 microbiological contaminants.

Although these water-borne contaminants pose a negative impact and risk on the safety of drinking water and the human population, they also greatly impact all organisms found in the environment. Pollution has been associated as a primary factor in multiple large-scale disturbances to both terrestrial and aquatic populations. These perturbations include the bleaching of coral reefs; mass die-offs of dolphins and seals; unusual phytoplankton blooms; cancer epizootics, or epidemics, in fish; and population decline of amphibians. Specific pollutants that have frequently been cited as being of concern include chlorinated organic pollutants such as PCBs, DDT, and acid rain (Sarokin and Schulkin 1992). However, many of the other contaminants cause problems to aquatic organisms, whether those problems are physiological effects or population decline. Exposures in the aquatic environment are particularly problematic, because aquatic organisms are subject to continual, unrestricted life-cycle exposures (Daughton and Ternes 1999).

One category of contaminants with potential detrimental effects on aquatic life is pharmaceutical drugs. The active pharmaceutical ingredients (APIs) in medications can be released into the environment and be present in low, but detectable, concentrations. This occurs mainly from patient excretion that travels into wastewater treatment plants (WWTP) or domestic septic systems. Some of these pharmaceutical agents are not entirely degraded during sewage treatment and therefore, are released into the environment via wastewater effluent. APIs can also enter the environment from farms and domestic animals, since the pharmaceuticals include both human and veterinary drugs (including antibiotics) (Cunningham et al. 2006). Veterinary pharmaceuticals that are used in animal agriculture and feeding operations are released to the environment with animal wastes through overflow or runoff (Meyer et al. 2000). Currently, little is known about the extent of environmental occurrence and all of the potential health effects to aquatic organisms exposed to low levels of most of these pharmaceuticals designed to stimulate a physiological response in humans and animals (Kolpin 2002).

Important information on the fate and long-term effects of antibiotics in particular is still lacking. Antibiotics are chemicals, either naturally occurring or man-made, that fit into different classes such as quinolones, β-lactams, tetracyclines, macrolides, and sulfonamides (Kümmerer 2009). Examples of antibiotics found in the environment include amoxicillin, chlortetracycline, ciprofloxacin, clarithromycin, enrofloxacin, gentamicin, norfloxacin, sufamethoxazole, and trimethoprim, among many others (Batt et al. 2007). One category of antibiotics found in the environment is the fluoroquinolones (FQs), which are a class of synthetic antibacterials with broad-spectrum antibiotic properties. Their environmental fate and effects are of interest, because they are

frequently used in human and veterinary medicine (Knapp et al. 2005) and are measureable in detectable concentrations in the environment (see Table 1).

Table 1. Concentrations of fluoroquinolones in the environment

Compound	Concentration	Conditions	Source
Ciprofloxacin	0.039 μg/L	Downstream of WWTP	Haggard et al. 2006
	0.043-0.076 μg/L	Downstream of WWTP	Batt et al. 2006
	3-87 μg/L	Hospital effluent	Hartmann et al. 1998
	9.3-11 ng/L	Qiantang River, China	Tong et al. 2011
	1.2-10.9 x 10^3 ppt	Hospital effluent	Duong et al. 2008
	328 ppt	Hospital effluent	Takasu et al. 2011
	850-2000 ng/L	Hospital effluent	Brown et al. 2006
Enrofloxacin	0.02 μg/L	U.S. streams	Kolpin et al. 2002
	10.5-18.7 ng/L	Qiantang River, China	Tong et al. 2011
	Up to 25,000 ng/L	Lakes near Hyderabad, India	Fick et al. 2009
	Up to 30,000 ng/L	Isakavagu-Nakkavagu rivers, India	Fick et al. 2009
Norfloxacin	0.06-6.06 x 10^6 ppt	Shrimp pond	Le and Munekage 2004
	7-12.9 ng/L	Qiantang River, China	Tong et al. 2011
	1.5-15.2 x 10^3 ppt	Hospital effluent	Duong et al. 2008
	4.62-2560 ppt	Hospital effluent	Takasu et al. 2011
Ofloxacin	0.109 μg/L	Downstream of WWTP	Haggard et al. 2006
	45.7-51.6 ng/L	Qiantang River, China	Tong et al. 2011
	185-782 ppt	Canal	Takasu et al. 2011
	Up to 146 ng/L	Ebro River, Spain	Gros et al. 2007
	Up to 23,500 ng/L	Residential effluent	Brown et al. 2006
	Up to 35,500 ng/L	Hospital effluent	Brown et al. 2006
Oxolinic acid	0.01-2.5 x 10^6 ppt	Shrimp pond	Le and Munekage 2004

When the antibiotics are detected in the environment, their concentrations are in the ng/L-µg/L (ppt-ppb) range. Although parts-per-billion (µg/L) concentrations may not pose acute risk, there could be nontarget aquatic organisms that are sensitive to these low concentrations (Daughton and Ternes 1999). There is particular concern about FQs because they have been shown to be genotoxic in hospital wastewaters (Hartmann et al. 1998), and they are leading to increased antibacterial resistance of pathogenic bacteria in clinical settings (Molbak 2004). Both ciprofloxacin and levofloxacin are important for medical use, but enrofloxacin is of greater interest in the environment since it is used in agriculture (Witte 1998). Due to the role of symbiotic bacteria in allowing herbivory, environmental antibiotics, such as enrofloxacin, may negatively affect herbivores.

Herbivory

Herbivory is a type of consumption in which the organism mainly feeds on autotrophs, like plants, algae, and photosynthesizing bacteria. There are multiple vertebrate species that utilize this feeding strategy, including mammals, birds, reptiles, fish, and amphibians. These organisms are anatomically and physiologically adapted to eat plant-based foods in order to fulfill their nutritional needs. Herbivores are adapted to their feeding mode through multiple morphological and physiological modifications (Adey and Loveland 2007). Modifications that can be found in herbivores include grinding and shearing teeth, elongated digestive tracts, and enlarged regions of the gut in which microbial symbionts live. These microbial symbionts help the organism solve the problem of the difficulty in digesting plant cell walls (Mackie 2002).

The cell walls of most plants, including algae, contain cellulose as the major component in the support structure. Cellulose poses difficulty, though, because

vertebrates do not produce cellulose, which is needed to break down the cellulose. Therefore, vertebrate herbivores have to depend on a gut microflora of cellulolytic bacteria or microbes to break down and digest the plant food (Zug et al. 2001). Although the walls of most aquatic plants are not as thick and lignified as those of woody plants, the walls of marsh plants still contain lignin and break down slowly. Even completely submerged aquatic plants and algae are very difficult to digest in comparison with animal cells. There are some herbivores that can simply crush the cells and digest the contents, but others have specially modified gastrointestinal (GI) tracts, which house microbes that assist in breaking down the cellulose (Adey and Loveland 2007).

Herbivorous amphibians face the same problem of lacking the ability to break down and digest the cellulose in the plant material that makes up their diets. They are the least studied group of herbivores as to what exact modifications help them solve this problem. Pryor and Bjorndal (2005) conducted a study on bullfrog tadpoles to attempt to answer the question of whether herbivorous amphibians exhibit the same characteristics as other herbivorous vertebrates. From the results of the study, they concluded that the physiological traits in bullfrog tadpoles match those found in the herbivorous vertebrates that depend on fermentative digestion in the hindgut. These specialized physiological features include a long, voluminous gut and enlarged colon that is home to many fermentative symbionts. The relative gut length of the bullfrog tadpoles was measured and found to be 1.5-8.5 times greater than gut lengths of 13 species of nonherbivorous anuran larvae studied by Altig and Kelly (1974). This supports that the gut lengths of tadpoles are correlated with their diets. Herbivorous species of tadpoles have much longer guts than the carnivorous or omnivorous species (Altig and Kelly 1974).

Herbivorous amphibians are among those organisms that have microbes in their GI tracts to help digest the food they consume. Without this microflora, an amphibian would most likely not be able to eat and digest enough plant material to survive on a strict herbivorous diet (Zug et al. 2001). The tadpole larvae of most of the anuran species feed on herbivorous diets of plants, plant-based detritus, periphyton, and phytoplankton (Sanderson and Kupferberg 1999). They gather food from all the levels of the water column, including grazing on the bottom sediments, filtering midwater phytoplankton, and skimming algae or vegetation floating on the surface. A particular species will specialize in feeding from a certain portion of the water column (Zug et al. 2001).

The GI tract is a specialized tube split into distinct anatomical regions from the mouth to the anus. Herbivorous reptiles, birds, and mammals normally have enlarged or elongated digestive tracts with fermentation chambers in the foregut or hindgut containing the microbial symbionts (Mackie 2002). This microbial community could include all the major domains of microbes, such as Bacteria, Archaea, and Eucarya, as well as viruses (Woese et al. 1990). High population density, wide diversity, and very complex interactions characterize the community. The bacteria, protozoa, and anaerobic fungi inhabiting the GI tract participate in mutualistic fermentative digestion. Although the carbohydrate polymers found in plant cell walls cannot be digested by most animals, they can be hydrolysed and fermented by the microbes living in the gut. The by-products of the fermentation are then utilized as an energy source by the host animal (Mackie 2002). For some herbivores, all or most of their daily energy needs are fulfilled by this symbiotic fermentation (Bjorndal 1997).

There are certain requirements that herbivorous organisms normally must meet in order to keep the gut microflora alive and functional. It seems that maintaining a constant, elevated body temperature is necessary to have gut microflora for many of the herbivorous species of vertebrates. Amphibians are ectothermic poikilotherms and, therefore, do not maintain a constant body temperature. This poses a question of how they efficiently keep their resident microflora alive and functional. Other requirements include constant food supply, slow passage of food through the gut to allow adequate time for bacterial breakdown, an anaerobic gut environment, regulation of the gut pH level, and removal of the waste by-products resulting from the fermentation (Zug et al. 2001).

Obligate herbivory does not exist in adult amphibians (Zug et al. 2001). This means that a frog begins its life feeding on an herbivorous diet in its larval stage and ends up switching to a carnivorous diet in the adult form. This is one of the many changes that occur during metamorphosis, the time when the aquatic larva must undergo major morphological changes to prepare for a primarily terrestrial lifestyle. In frogs, almost every organ is remodeled in some way, including hindlimbs and forelimbs, skull, teeth, tongue muscle, and intestine. The larval intestine with numerous coils to digest plant matter is transformed into a shorter intestine. It changes from a long spiral gut with intestinal symbionts to a short gut with protease enzymes (Gilbert 2010).

The remodeling of the intestine during metamorphosis is a very complex process. The morphological changes that occur during it are more drastic than those that occur in both the liver and brain. The adult amphibian intestine resembles the intestine found in higher vertebrates with elaborate connective tissue and muscles. On the other hand, the

structure of the tadpole intestine is much longer but simpler. It consists of only one layer of columnar epithelium surrounded by thin layers of muscles with a small amount of intervening connective tissue (Shi 1999). At the onset of metamorphic climax (NF stage 58 in *Xenopus laevis*; Nieuwkoop and Faber 1994), the long larval intestine begins to shorten suddenly. This continues until metamorphosis is over, leaving a much shorter, completely restructured intestine. The major structural changes that occur between larval and adult intestines reflect the changes in physiological functions between the herbivorous tadpoles and carnivorous frogs (Shi 1999).

Purpose of Study

The purpose of this study is to investigate the effects of the antibiotic enrofloxacin on the gastrointestinal tract of herbivorous tadpoles. This will be accomplished by examining the cellular structure of the intestines of *Xenopus laevis* exposed to enrofloxacin. Before the effects of enrofloxacin can be evaluated in an experiment, a descriptive study of normal GI tract ontogeny in *Xenopus laevis* tadpoles is necessary. Enrofloxacin is hypothesized to have effects on intestinal epithelial structure, as bacteria adhere to epithelial cells in the GI tract and, therefore, influence the GI tract structure (Bengmark 1998).

CHAPTER II

MATERIALS AND METHODS

Descriptive Study

Individual *Xenopus laevis* tadpoles (NF stages 53, 55, 56, 59, and 66) were obtained from Nasco (enasco.com). Tadpoles were anesthetized in 400 mg/L Ethyl 3-aminobenzoate (MS-222), and intestines were removed using micro dissection scissors and forceps, fixed in Bouin's fixative for 3 days, and cleared with 70% ethanol. GI tract tissues were processed using histology protocols according to Presnell and Schreibman (1997). The tissues were embedded in wax blocks by first moving the tissues to increasing percentages of alcohol, then SAFECLEAR tissue clearing agent (Protocol Cat# 314-629), and then into paraffin wax under vacuum. After trimming the paraffin blocks around the tissues, the blocks were sectioned at 12 μm. The ribbons were mounted on slides by using a warm water bath with gelatin, and set to dry on a slide warmer.

Slides were stained using Hematoxylin and Eosin (Presnell and Schreibman 1997) and analyzed to examine the transformations of the GI tract throughout development of the tadpoles. Multiple (n=7-8) intestinal epithelial cell height measurements were taken for each individual. Epithelial cell density measurements (n=5-8) were also taken by

counting the number of epithelial cells in 2.47 μm. Because single tadpoles were analyzed at each stage, statistical analyses could not be conducted.

Experimental Study

Tadpoles, ranging from NF stage 52 to 65, were divided into 3 groups, each containing 9 individuals (3 at stage 53-58, 3 at stage 59-62, 3 at stage 63-66). Individual tadpoles were maintained in one-liter beakers with 800 mL spring water and exposed to either enrofloxacin (Baytril) (2.5 μg/mL for "low dose" or 25 μg/mL for the "high dose" group) or Ringer's solution ("control" group). The water in the beakers was changed and the doses re-administered every other day. Tadpoles were fed frog brittle on the days the water was not changed.

After 10 days of exposure, tadpoles were anesthetized and staged according to Nieuwkoop and Faber (1994). After removing intestines, fecal matter was extracted by pressing GI tract using 2 pairs of forceps, weighed (.001g-0.005g), and then placed into sterile 1.5 mL tubes with 50 μL sterile phosphate buffered saline (PBS), prepared by mixing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and adjusting to a final pH of 7.4. The tubes were vortexed, and each sample was diluted 1:100 and 1:10,000 with PBS, then spread onto sterile LB agar plates, prepared by mixing 20g dehydrated LB broth, Lennox (Fisher Scientific) and 15g granulated agar (Difco) in 1 L purified water, then pouring into plastic petri dishes. After spreading the samples, the plates were incubated at 25°C for 72 hours. Bacteria were quantified by counting the number of bacterial colonies growing on each plate. An analysis of variance (ANOVA) was conducted to compare number of bacterial colonies in each treatment group for each dilution.

After removal of fecal matter, GI tracts were placed into Bouin's fixative for 3 days and then processed using histology protocols according to Presnell and Schreibman (1997). Slides were prepared, stained, and analyzed. Multiple intestinal epithelial cell height (n=7-8) and epithelial cell density measurements (n=5-8) were taken from the mid region of the intestine (duodenum) and the caudal, coiled section (ileum). A two-factor ANOVA (with Tukey's post-hoc test) was used to compare tadpoles exposed to the different doses for tadpoles in different stage categories.

CHAPTER III

RESULTS

Descriptive Results

At NF stage 53 in the development of *X. laevis* tadpoles, the intestinal structure consists of a columnar lining epithelium, a single layer of epithelial cells surrounding the lumen (Figures 1 and 2). By NF stage 56 in the duodenum, the epithelium starts forming folds, and submucosa begins to form surrounding the epithelium (Figure 1). There is only one major epithelial fold that projects into the intestinal cavity present in the duodenum at NF stages 56 and 59 (Figure 1). At NF stage 59, both the duodenum and ileum sections show development of submucosa and a thin layer of external muscle starting to develop (Figures 1 and 2).

After metamorphosis (NF stage 66), the duodenum contains a layer called the muscularis externa, which consists of a thick layer of inner circular smooth muscle and a thinner layer of outer longitudinal smooth muscle (Figure 1). Also, the epithelium has become much more intricately folded into many crypts and villi. By this stage, the ileum section of the intestine is no longer coiled at all. Although not as thick as in the

duodenum, there is a layer of submucosa and both inner circular muscle and outer longitudinal muscle layers (Figure 2).

Experimental Results

Epithelial cell height and density measurements for untreated tadpoles at NF stages 53, 55, 56, 59, and 66 are shown in Table 2. For epithelial cell height measurements of the treated tadpoles (Table 3), significant differences were found between doses ($p=0.014$), between stage categories ($p=0.010$), and with interaction between dose and stage ($p=0.035$; see Figure 3). For epithelial cell density, no significant differences were found between doses ($p=0.848$), between stage categories ($p=0.208$), or with interaction between dose and stage ($p=0.866$).

The agar plates containing contents from the GI tracts of the treated tadpoles were photographed after 72 hours of incubation (Figure 4). The types of bacteria growing on the plates from the control tadpoles were different from the tadpoles exposed to low and high doses of enrofloxacin, demonstrated by the change in appearance and color of the bacterial colonies. Bacterial colonies were counted on each of the plates. Mean numbers of colonies counted were not different for the different treatment groups at the 1:100 dilution ($p=0.879$, Figure 5) or the 1:10,000 dilution ($p=0.690$, Figure 6).

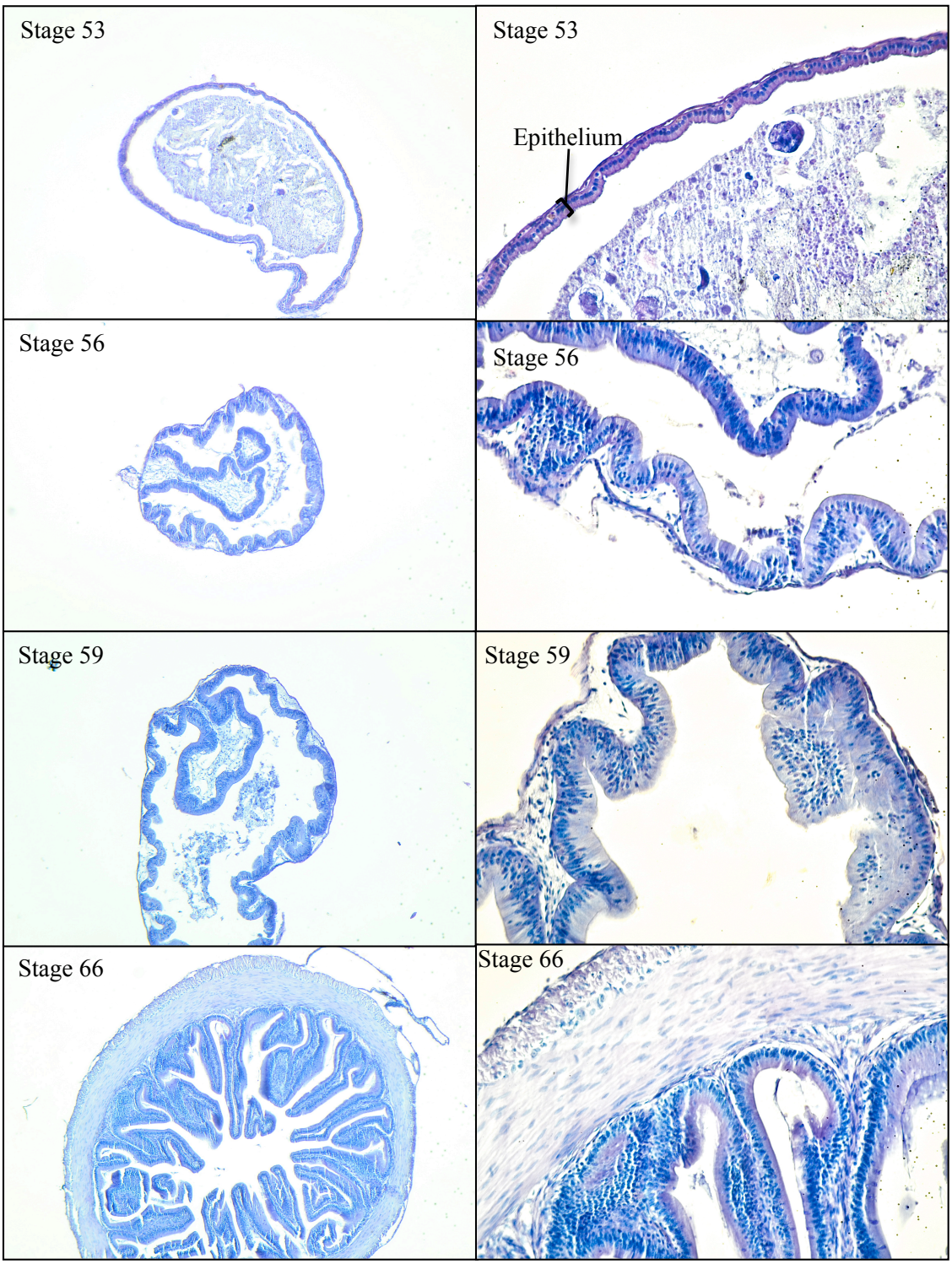

Figure 1. Cross sections of duodenum at NF stages 53, 56, 59, and 66 photographed at 10x magnification (left column) and 40x magnification (right column). Note the single layer of epithelial cells at NF 53 that starts forming folds at NF 56 and is highly folded into crypts and villi after metamorphosis (NF 66). Submucosa and muscle layers develop throughout the stages surrounding the epithelium.

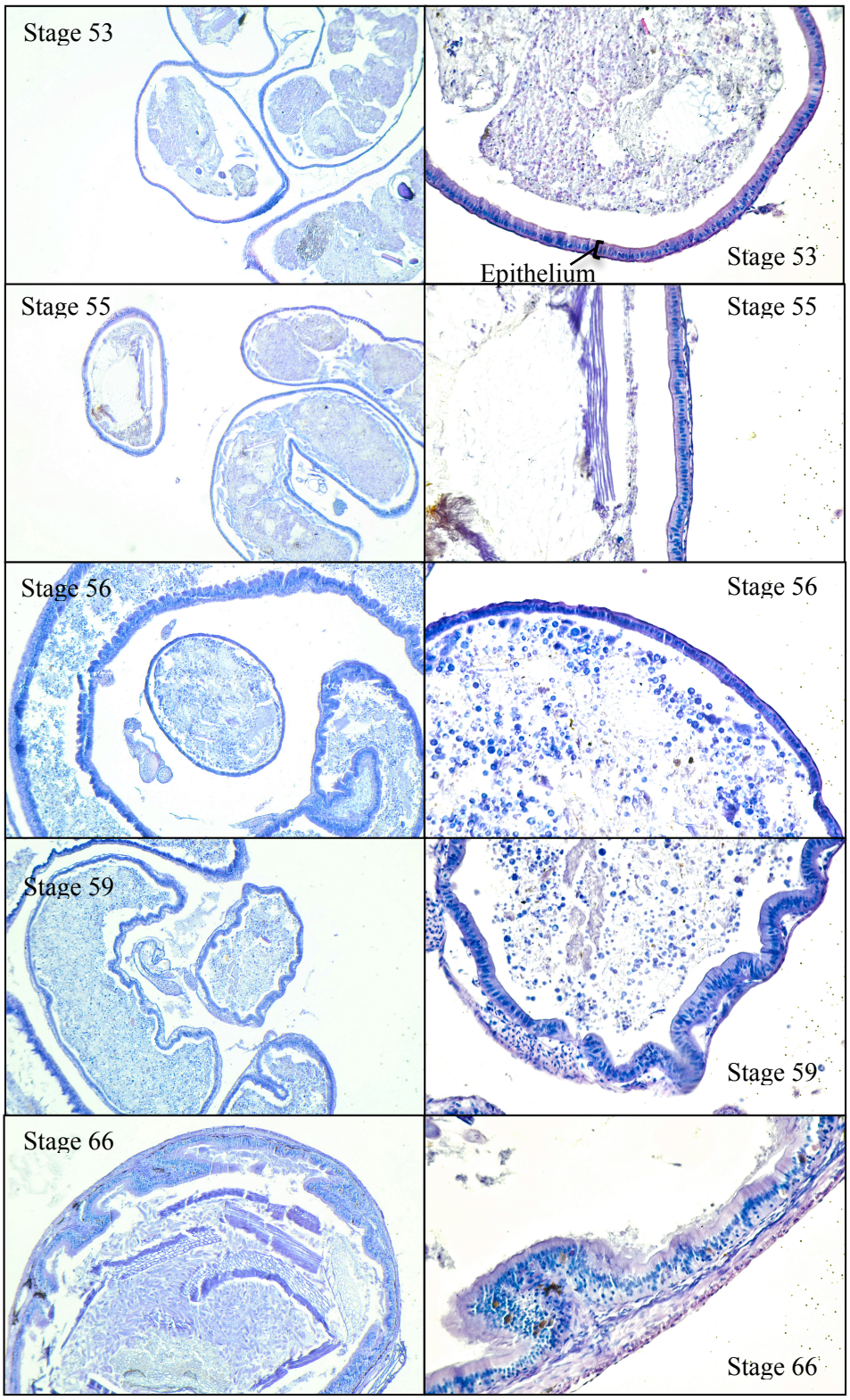

Figure 2. Cross sections of ileum at NF stages 53, 55, 56, 59, and 66 photographed at 10x magnification (left column) and 40x magnification (right column). Note the single layer of epithelial cells (NF 53-56) that forms folds at NF 59 and thin layers of submucosa and muscle at NF 59 and 66. The intestine is no longer coiled after metamorphosis (NF 66).

Table 2. Mean epithelial cell height and density measurements (±1SE) for duodenum and ileum sections from tadpoles at NF stages 53, 55, 56, 59, and 66

Stage	Duodenum Height	Ileum Height	Duodenum Density	Ileum Density
53	18.53 ± 0.539	18.17 ± 1.10	1.909 ± 0.116	1.966 ± 0.106
55		19.94 ± 0.828		2.140 ± 0.170
56	29.99 ± 1.39	21.52 ± 1.59	2.140 ± 0.116	2.082 ± 0.106
59	42.34 ± 1.37	26.46 ± 1.07	2.487 ± 0.206	2.313 ± 0.192
66	28.93 ± 1.68	40.58 ± 2.65	2.892 ± 0.106	2.834 ± 0.216

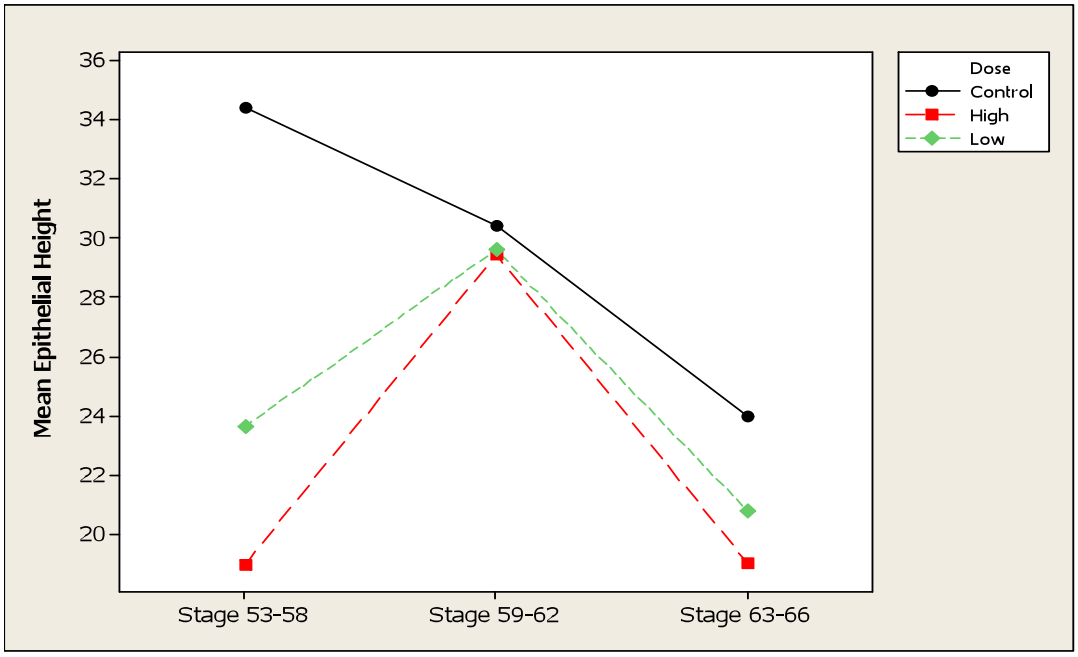

Figure 3. Interaction plot of dose and stage of tadpole for mean epithelial cell height

Table 3. Mean epithelial cell height and density measurements (±1SE) for tadpoles exposed to control, low, and high doses of enrofloxacin grouped into different stage categories

	Control	Low	High
Stage 53-58 Height	34.40 ± 2.51	23.64 ± 0.911	18.99 ± 1.09
Stage 59-62 Height	30.41 ± 1.09	29.64 ± 1.32	29.46 ± 0.786
Stage 63-66 Height	23.99 ± 1.40	20.82 ± 0.911	19.05 ± 0.529
Stage 53-58 Density	2.105 ± 0.151	1.909 ± 0.116	1.872 ± 0.106
Stage 59-66 Density	2.267 ± 0.103	2.227 ± 0.070	2.227 ± 0.067

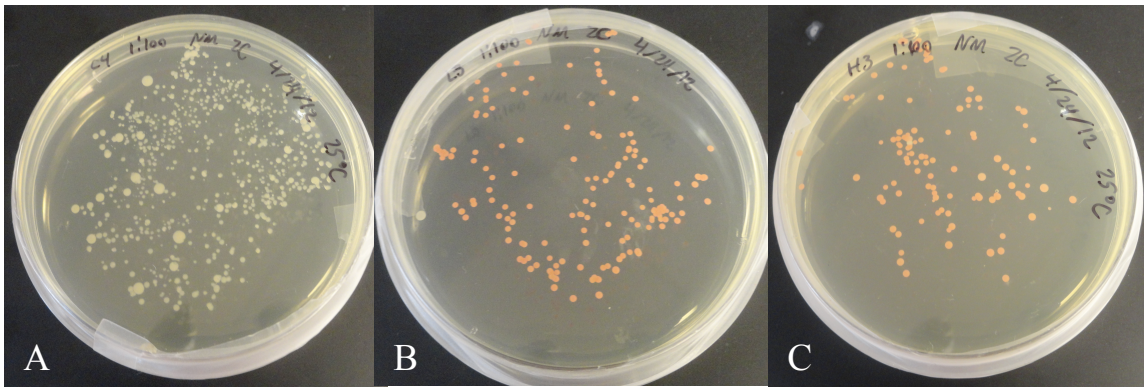

Figure 4. Bacterial colonies growing on agar plates spread with GI tract contents (1:100) from control tadpoles (A) and tadpoles exposed to low (B) and high (C) doses of enrofloxacin after 72 hours of incubation at 25°C

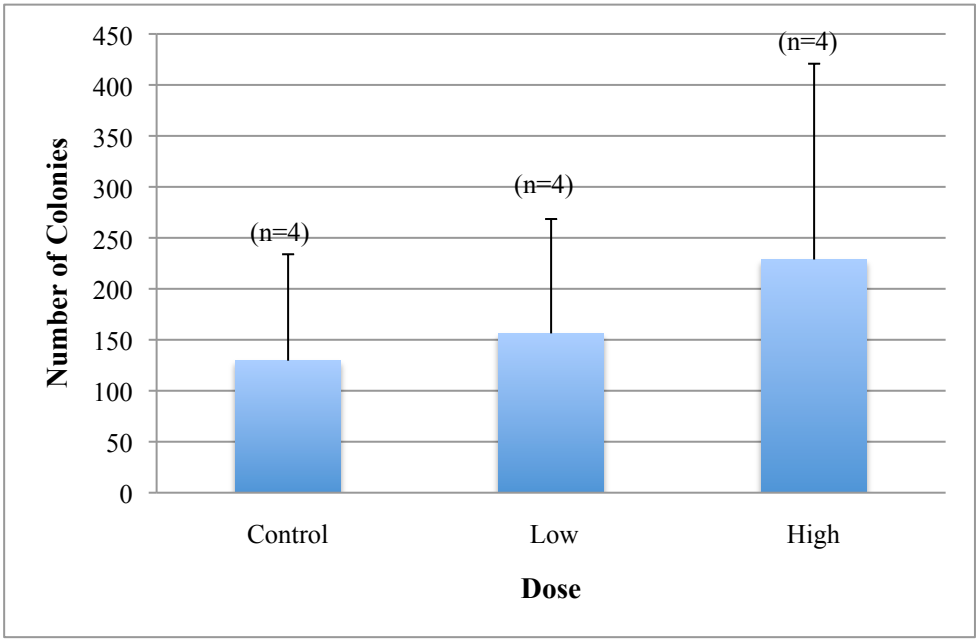

Figure 5. Mean number of bacterial colonies counted (+1SE) on agar plates spread with GI tract contents (1:100 dilution) from control tadpoles and tadpoles exposed to low and high doses of enrofloxacin after 72 hours of incubation at 25°C

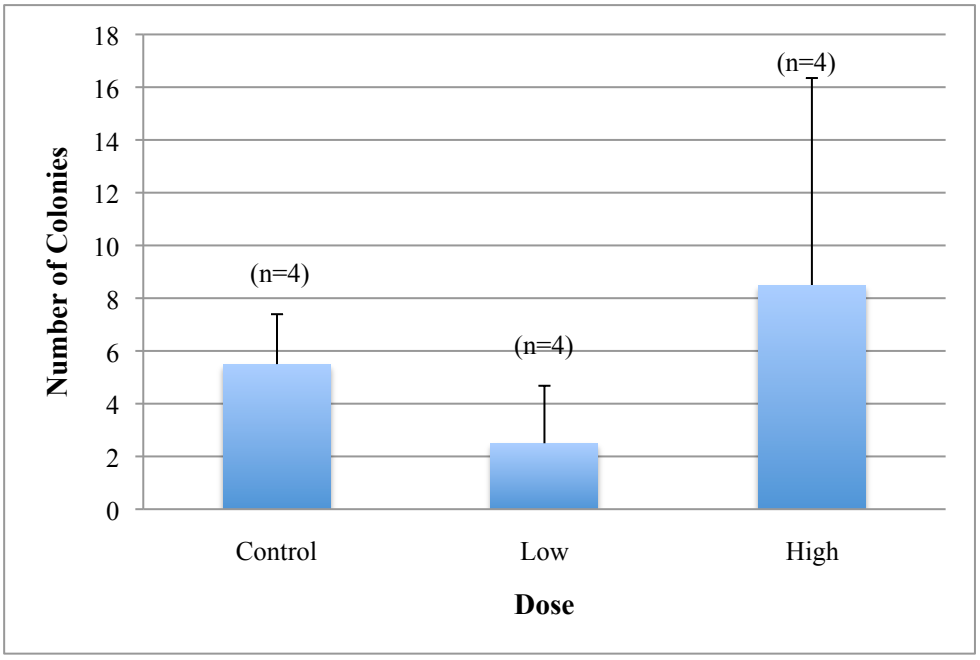

Figure 6. Mean number of bacterial colonies counted (+1SE) on agar plates spread with GI tract contents (1:10,000 dilution) from control tadpoles and tadpoles exposed to low and high doses of enrofloxacin after 72 hours of incubation at 25°C

CHAPTER IV

DISCUSSION

In the descriptive study, changes were observed in intestinal structure throughout development of *X. laevis* tadpoles characterized by shortening of the intestine, increased epithelial cell height and folding of the epithelium into crypts and villi, and in the experimental study several effects of the antibiotic enrofloxacin on the GI tracts of the tadpoles were observed. These changes include decreased epithelial cell height and altered intestinal microflora, suggesting that exposure to enrofloxacin can have effects on both intestinal structure and function in *X. laevis* tadpoles.

The drastic morphological remodeling of the intestines of *X. laevis* tadpoles throughout metamorphosis was evident in both the appearance of the tissues when removed and the histology. Major changes in histological structure were observed throughout the tadpoles' stages of development. While the GI tract transformed from a simple, coiled tube into a much shorter, straight gut, the results displayed that thicker, more complex walls were developing. Epithelial cell heights in the duodenum increased between NF stages 53 and 59, but then were reduced by NF stage 66 after metamorphosis. Only one involution, or fold, was evident in the duodenum before metamorphosis, called

the typhlosole, as described by Marshall and Dixon (1978). Although the epithelium was thinner, the walls became more complex with thicker muscle layers and an epithelium highly folded into troughs and ridges that resemble the anatomy of a typical adult vertebrate intestine. These findings are very similar to the observations of Schreiber et al. 2005, who reported that the *X. laevis* intestine shortened by 76% from the beginning of metamorphosis at NF stage 60 through the end by NF stage 66. They also reported thickening of the epithelium and muscle layers and a temporary heaping of epithelial cells into many layers at the metamorphic climax. By the end of metamorphosis, the epithelium was, once again, a single-cell layer folded into crypts and villi.

Tadpoles exposed to both the low and high dose of enrofloxacin exhibited significant decreases in intestinal epithelial cell height when compared to the control tadpoles. The extent of these decreases was dependent on stage of the tadpoles. Exposure to enrofloxacin had the most significant effect on epithelial cell height in the earlier stage tadpoles (NF stage 53-58). There also appeared to be effects of enrofloxacin on the gut microflora of the tadpoles. Tadpoles exposed to enrofloxacin exhibited a different type of bacteria in the contents of the GI tract than the control tadpoles, although the bacteria type was not identified.

The finding that tadpoles exposed to enrofloxacin exhibited decreases in intestinal epithelial cell height suggests that exposure to enrofloxacin led to thinning of the intestines. A similar finding was observed in chicks given penicillin, as their small intestine appeared thinner than the intestines of the controls (Coates et al. 1952). Indeed, penicillin reduces the weight of the small intestine in chicks, leading the authors to suggest that the gut is thickened in conventionally kept animals to serve as a defense

against absorbing bacterial toxins or other detrimental microbial products (Coates et al. 1955). Thus, the addition of an antibiotic would remove some undesirable microorganisms, and the gut would be thinner without the typical pathological reaction. This could be one potential explanation for decreased epithelial cell height and thinner intestines.

An alternative explanation for the epithelial thinning is that it is caused by the antibiotic eliminating the microbial community associated with the GI tract. Results from the current experiment do not support this explanation, however. The types of bacteria in the contents obtained from the GI tracts of the tadpoles exposed to enrofloxacin appeared to be different from the bacteria from the controls. However, the fact that bacteria was discovered in the gut contents of the exposed tadpoles demonstrates that the antibiotic did not completely eliminate the intestinal microflora. The hypothesis that exposure to enrofloxacin would reduce the amount of gut microflora was not supported. Rather, enrofloxacin caused a change in the type of microorganisms observed.

The conclusion that different types of bacteria were discovered growing in the GI tracts of the exposed tadpoles is based on qualitative observations of the bacterial colonies. It is conceivable that enrofloxacin eliminated the normal primary microflora, therefore allowing other types of bacteria to grow and thrive without competition. Further identification of the types of bacteria in the gut contents of each tadpole was not conducted for this experiment. Also, this experiment only included aerobic or facultative anaerobic bacterial species, since conditions for the successful growth of obligate anaerobic species were not available. Future studies should include both the obligate anaerobic species and more detailed identification of the specific types of bacteria

obtained to more accurately observe the effects of enrofloxacin on the gut microflora of the tadpoles.

The results of this study show that enrofloxacin can affect the normal structure and function of *X. laevis* intestines. In this study, enrofloxacin was found to have effects on both intestinal structure, with decreases in epithelial cell height, and microflora, with elimination of some of the normal resident bacteria. Since enrofloxacin is a veterinary antibiotic frequently used in animal agriculture, it is released into the environment with animal wastes through runoff and has been discovered in detectable concentrations in various freshwater ecosystems worldwide (e.g. Kolpin et al. 2002, Fick et al. 2009, and Tong et al. 2011). Therefore, populations of *X. laevis* or other anuran species are likely exposed to potentially harmful concentrations of enrofloxacin and other antibiotics found in the environment.

APPENDIX

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: Drew Crain

Email Address: drew.crain@maryvillecollege.edu

Date: April 10, 2012

Species to be used: *Xenopus laevis*

Age of animals: Stage 56 through metamorphisis

Number of animals in study: 30

Brief description of use (teaching or research): Tadpoles will be exposed to the antibiotic Baytril in Bio414: Developmental Biology lab.

Duration of use: 1 month

Location of animals (building and room) : 114 Sutton

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Dr. Crain	981-8238	981-8238	981-8238

What will happen to the animals at the end of the use? If euthanasia is required, state the methods.
Euthanasia with 400 mg/L MS-222.

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

Maryville College IACUC Approval Number: 2012-04
Date Approved: 4/12/12
Signed: Irene Greenwood

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