EFFECTS OF NITRATE ON TISSUE LEVEL BIOMARKERS IN POST-METAMORPHIC XENOPUS LAEVIS

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biology.

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ABSTRACT

Amphibian populations have been declining globally since the 1980's. Threats putting the populations at risk include global warming, habitat or land-use changes (including increased nitrogen application), emerging infectious disease, invasive and exotic species, ultra-violet radiation, and toxicants such as those used in agricultural practices. The effect of these stressors on amphibian population include not only decline in abundance but also developmental deficiencies in growth rate, behavioral, and morphological abnormalities.

The study presented in this paper, focuses on potential tissue level biomarkers of nitrogen exposure. The use of a chronic exposure during multiple critical developmental periods at ecologically relevant levels of nitrate will produce data that reflects the resilience or the susceptibility of the frog, *Xenopus laevis*. Measures of fluctuating asymmetry, hematocrit, body condition, and metamorphic data were collected and analyzed to determine the effect of nitrate on *Xenopus laevis*.

The results of this study largely determined no significant difference between treatments for the bioindicators measured when Xenopus laevis were exposed to nitrate from hatching to two-months post metamorphosis. These suites of tests are important to literature for two primary reasons 1) substantiation of the water quality test reinforces the quality of the test and tight controls in place 2) the length of chronic exposure in a multi-factorial test.

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LIST OF ABBREVIATIONS

AFO	Animal Feeding Operations
AA	Antisymmetry
cm	Centimeters
CO(NH2)2	Urea
DA	Directional asymmetry
DDT	Dichlorodiphenyltrichloroethane
DS	Developmental stability
E2	Estrogen
EDC	Endocrine-disrupting chemicals
EPA	Environmental Protection Agency
FA	Fluctuating Asymmetry
g	Grams
g1	Skewness
g2	Kurtosis
GLM	General linear model
GSI	Gonad somatic index
GSI	Gonadal Somatic Index
GW	Gonad weight
H&E	Hematoxylin and Eosin
HAI	Histology Scoring Index
Hb	Hemoglobin
hCG	Human chorionic gonadotropin

LIST OF ABBREVIATIONS (continued)

HSD	Tukey's Studentized Range Test
IU	International Units
K	Body Condition
LD50	Lethal Dose, fifty percent of the tested population
LOAEL	Lowest-observable-adverse-effect-level
LSI	Liver Somatic Index
MCL	Maximum Containment Level
ME	Measurement error
metHb	Methemoglobin
mg	Milligrams
mg/L	Milligrams per liter
MHW	Moderately Hard Water
mm	Millimeters
N	Nitrogen
N_20	Nitrous oxide
NaNO3-	Sodium Nitrate
NH4+	Ammonium
NO	Nitric oxide
NO2-	Nitrite
NO3-	Nitrate
NOAEL	No-observable-adverse-effect-level

LIST OF ABBREVIATIONS (continued)

PCB	Polychlorinated biphenyls
SAS	Statistical Analysis System
SD	Standard deviations
SVL	Snout-vent length
T	Testosterone
TW	Total body weight
WHO	World Health Organization
Y	Mean

CHAPTER 1

INTRODUCTION

1.1 Nitrate Effects on Global Amphibian Decline

Amphibians survived the last great mass extinction, the Cretaceous-Tertiary boundary (~65 Mya). Research suggests that world ecosystems may be entering a sixth great mass extinction targeting the class Amphibia (Wake, 2008). Focus on the declining abundance and biodiversity of amphibians intensified since the late 1980s and is documented in diverse habitats on multiple continents (Rouse, 1999). The current estimated rate of amphibian extinction is 211-times the background rate of extinction (Wake, 2008).

Primary threats for amphibians, more than 6,300 species, (Wake, 2008) include: climatic change, habitat degradation and fragmentation, invasion of exotic species, viral and fungal pathogens, increased UV radiation, and industrial and agricultural chemical contaminants. Efforts to identify the causes of amphibian decline have yielded an extensive list of environmental toxicants that produce a range of deleterious effects. Toxic agricultural and industrial chemicals such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), pesticides, and industrial effluent are examples. Recent work has focused on chemical compounds termed “endocrine-disruptors” which cause estrogenic, anti-estrogenic, thyroid-disrupting, androgenic, and anti-androgenic effects that may seriously disrupt fertility.

Research on environmental nitrates as a suspected factor in the global amphibian declines has increased (e.g., Guillette et al. 2005; Hayes et al. 2006; Rouse et al. 1999). The use of amphibians as bioindicators for the effects of nitrates includes both field and laboratory studies that report a wide range of effects including altered behaviors in movement and feeding, disruption of gonad function and steroid synthesis pathways, gonad atrophy, and reduction in sperm count and altered sperm morphology (Edwards et al. 2006). Amphibian models represent more than 45% of the peer-reviewed literature in toxicology-oriented studies that assess the environmental impact of chemicals in the last ten years (Mann and Bidbwell, 1999). Three genera (*Rana*, *Bufo*, and *Xenopus*) comprise 80% of the amphibian toxicological literature (Mann and Bidwell, 1999). Amphibians are proposed as ideal indicator species of environmental stressors due to their global presence, long phylogenetic history, two life phases (aquatic and

terrestrial), and sensitivity to environmental perturbation. The complexity of their life cycle increases opportunities of exposure to a variety of contaminants. Amphibians are the highest vertebrate group to retain a "naked" egg (embryo is sheltered by a gelatinous covering), the only vertebrate group (applies to the majority of species of amphibia) to have an aquatic larval stage, and transition at metamorphosis to a terrestrial phase of adult life. Hormone-regulated developmental stages while in an aquatic environment provide a means to measure the effects of endocrine-disrupting chemicals. Other physiological features such as externalized gills for respiration in the larvae stage and an adult skin that is a permeable organ used for respiration and water-balance also make amphibians interesting model species for such exposure studies.

Recent field observations of amphibian deformities brought concerned public attention to the impacts of environmental perturbations on species. These deformities are particularly associated with nitrate-based environmental contamination from surrounding agricultural runoff and effluent (Rouse, 1999). Nitrate-based effects may not be the only cause of some of the observed deformities because recently a trematode was also implicated (Stopper et al., 2002). One of the first reports of deformities was from a south-central Minnesota rural wetland area in the summer of 1995. The following is a recount from Dr. Michael Lannoo (2008) of Muncie Medical College, in his book, Malformed Frogs: The Collapse of Aquatic Ecosystems.

> *"While exploring a rural wetland, Cindy Reinitz and her junior high school students discovered a large number of northern leopard frogs (Rana pipens) that were having trouble jumping. When normal frogs with two good hind legs jump they maintain control in the air and land on their hands and their chest. But the leopard frogs these kids were finding had missing legs or missing parts of legs..."*

Following similar reports of amphibian deformities and loss of species in other geographical areas, a number of environmental impact analyses were conducted to examine the potential outcomes of agricultural activity on these species.

McCoy et al. (2008) examined the association between agricultural intensity in a habitat and the frequency of altered endocrine systems in amphibians. Chemicals such as pesticides, plasticizers, fungicides, and other synthetics that show disturbance of reproductive function, normal growth and development, and altered displayed behaviors are called endocrine-disrupting chemicals (EDCs). The EDCs are chemicals that mimic or interfere with hormone function,

particularly estrogen function but, in many cases, the link between a chemical and disruption is unclear (Jobling, 2006). The EDC target tissues include thyroid, retinoid, gonadal, and adrenal systems.

McCoy et al., (2008) assessed gonadal abnormalities/dysfunction in Bufo marinus from five sites ranging in agricultural use (intensity of agricultural impact) from zero to 97%. A dose-dependent response was observed between the frequency of intersex gonads, the number of abnormalities, and the increase in agriculture pressure (i.e., more abnormalities as agricultural intensity increased). Evidence of abnormal alterations in secondary sex traits included demasculinization of males resulting in reduced forearm width (McCoy et al., 2008) that interferes with amplexus, and/or feminization that presented as increased skin mottling. Steroid hormone concentrations and secondary sexual traits correlate with reproductive activity and success. Reproductive abnormalities and reduced reproductive success should contribute to amphibian population declines in highly contaminated and intense agricultural areas.

A number of studies reported negative impacts on amphibians exposed to high levels of insecticides with EDC actions. For example, DDT, an organochlorine similar to dieldrin, caused an increase in the mortality rate of a population of western spotted frogs (*Rana pretiosa*) in Oregon (Carey, 1995). Kirk (1988) indicated a suspected dosing of DDT to an area of 172,695 hectares of forest in three states (Idaho, Oregon, and Washington) at a rate of 0.7 kg/ha that caused mortality in *Rana pretiosa*. Residue traces of DDT were found in amphibians not associated with sites where DDT was applied, thus raising the concern of bioaccumulation between trophic levels.

Sublethal responses to toxicants may also produce effects such as reduced predator avoidance behavior and altered gonadal morphology and physiology. Hayes et al. (2006) suggested that, given the relative risks that EDCs are proposed to have on an array of wildlife populations, EDCs should be considered as a likely cause for the widespread population declines in amphibian populations worldwide. Hayes and co-workers documented EDC-associated changes in the anatomy, behavior, and reproductive system function of many vertebrate taxa (Hayes et al. 2006). Hayes et al. (2006) examined the effects on *Xenopus laevis* and *Rana pipens* of nine different EDCs including herbicides, pesticides, and fungicides that are used alone or in combination in United States Midwestern agriculture. The list of chemicals used includes atrazine, alachlor, nicosulfuron, cyfluthrin, λ- cyhalothrin, tebupirimphos, metalaxyl, and

propiconizole. They were evaluated in combination and alone during the period of larval growth and development for effects on physical status at metamorphosis, sex differentiation, and immune function. Larvae were reared (30 per tank) in 4 L of aerated 0.1% Holtfreter's solution and fed Purina rabbit chow. The chemicals were administered at low levels of 0.1 to 10.0 parts per billion. Based on this research, it was determined that the multiple mixtures produced damage to the thymus and increased plasma levels of the stress hormone corticosterone (Hayes et al. 2006). Also, for those exposed to cyfluthrin, tebpirimphos, and atrazine, size at metamorphosis (snout-vent-length) and body weight were reduced.

Berger (1989) noted a correlation between amphibian decline and the increase in environmental nitrate and ammonia use. Nitrate mixtures commonly seen in agricultural settings include perchlorate, sodium, ammonium, and potassium. In agricultural regions, nitrate is applied in both separate and mixed applications with other herbicides, pesticides, or fertilizers in either liquid or granular form. Deleterious effects in amphibians from sodium and potassium nitrate salts include reduced survival, delayed growth rates, and increased frequency of abnormalities (eg. Baker and Waights 1993; Johansson et al. 2001; Marco et al. 1999). Baker and Waights (1993, 1994) observed reduced feedings and weight loss in *Bufo bufo* and *Litoria caerula* tadpoles at concentrations of 40mg/L and 100 mg/L of sodium nitrate, respectively.

Meredith and Whiteman (2008) reported on the effects of nitrate (NaNO3-) in amphibian embryos (embryonic development) using three aquatic breeding amphibian species (*Ambystoma mexicanum*, *Hyla chrysoscelis*, and *Rana clamitans*) whose larvae may be exposed to chemicals from weeks to months during aquatic development. A static-renewal system was used to apply differing treatments of nitrate-N (NO3-): 0, 5, 10, 30, 60, 100, 300, and 500 mg/L. Developmental progress and lethal effects were recorded from Gosner stage 2 and Harrison stage 2 (larval period) through hatching stage by recording the time to hatch and the length at hatching. The study found no significant difference in mortality between treatments or species (Meredith and Whiteman, 2008). *Ambystoma mexicanum* displayed earlier time to hatching and smaller individuals from the 300 and 500 mg/L treatments.

Ortiz-Santalistra (2006) examined the influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians. Ortiz-Santalistra developed a static renewal experiment for the use of ammonium nitrate on embryonic and larval stages of anuran amphibians. Amphibian species tested included *Discoglossus galaganoi*, *Pelobates cultripes*, and

Bufo calamita. The species displayed varying sensitivity based on life-stage and development at time of exposure. Younger amphibians displayed a more acute response and there was a difference in response between the three species. After four days of exposure to 225.8 mg N-NO3NH4/L, 100% of *P. cultripes* died.

In a successive study, Ortiz-Santaliestra (2007) evaluated alteration in larval development and metamorphic development when nitrate and perchlorate were applied to southern Leopard Frogs (*Rana sphenocephala*). The test design involved a static renewal with complete replacement of water and chemicals twice a week over a 15-week period that concluded when all control larvae reached metamorphosis. Nitrate was administered at 0 mg and 100 mg NO-3/l as sodium nitrate (Sigma-Aldrich, St. Louis, MO, USA) to *R. sphenocephala* larva, which had developed past Gosner stage 25 (free swimming) in medium-hard reconstituted water. They determined that nitrate at a concentration of 100 mg/L reduced embryonic and larval survival and development (Ortiz- Santaliestra, 2007).

Johansson et al. (2001) determined that exposure of sodium nitrate of up to 5 mg N-NO3-/l in *Rana temporaria* produced a smaller size and a longer time to metamorphosis. Other evidence from Sullivan and Spence (2002) showed delayed metamorphosis of *X. laevis* exposed to 40 mg/L nitrate with no effect of nitrate on body size. This again indicates the importance of sensitivity and diversity in the responses among species. Sullivan and Spence (2002) also indicate that an increase in body weight at metamorphosis occurs in *X. laevis* tadpoles exposed to nitrate. Behavioral changes were observed in *X. leavis* exposed to continuous low doses of nitrate in a static renewal system from the free-swimming stage to metamorphosis. Nitrate exposure also delayed forelimb emergence and tail absorption (Sullivan and Spence, 2002).

Guillette and Edwards (2005) produced evidence that nitrate's complex mechanism of action included alterations in hormonal function. Various studies indicated EDC's ability to alter normal hormonal signaling during embryonic development and thereby permanently change adult reproductive system morphology, function, and reproductive behavior (Guillette et al. 1995; McLachlan 2001; Zala and Penn 2004). Because many components of the endocrine system are highly conserved, EDCs can affect normal physiological functions across diverse taxa (Crews and McLachlan 2004; McLachlan 2001).

Guillette and Edwards (2005) published: "Is nitrate an ecologically relevant endocrine disruptor in vertebrates?" with data indicating that the answer is yes. Using previous work and

particularly their studies of alligators in the wild, they showed that steroidogenesis and endocrine patterns are disrupted when nitrate and/or nitrite is introduced. In reviewing the literature, they also found evidence of a "dramatic reduction in testicular Leydig cell steroidogenesis at pharmacological doses of nitrate and nitrite and decreased responsiveness of Leydig cells in mice to gonadotropin stimulation" (Panesar, 1999)." Other species, such as bulls, exhibited reduced sperm motility, increased secondary sperm abnormalities, and degenerative lesions in the spermatocyte and spermatid germ layers of the testis, when given nitrate (100-250 g/day/animal), (Zraly et al., 1997).

Barbeau and Guillette (2007) studied the effects of low-dose nitrate exposure on the adult amphibian female reproductive system. They showed that brief (simulated pulsed) exposure to environmentally relevant concentrations (seven days at concentrations of 24.8 mg/L or 49.5 mg/L [NaNO3 – N]) of sodium nitrate influenced ovarian steroid synthesis and ovarian follicle size in adult *Xenopus laevis*. Ovarian stimulation to obtain follicle development over a five week course used 50 IU of pregnant mare serum gonadotropin followed three days later by 750 IU of human chorionic gonadotropin into the dorsal lymph sac. Exposure to nitrate occurred six weeks later to provide time for follicle development. Water and nitrate solution was changed every 24 hours. The follicles of *Xenopus laevis* exposed to nitrate exhibited an increased stage-4 diameter at both concentrations and a decreased stage 5 and -6 diameter at 49.5 mg/L concentration. The results indicate that anuran ovarian steroidogenesis and follicle size are modified by varying exposure concentrations. The higher exposure of nitrate, 49.5 mg/L, showed a decrease in later stages of follicle development (Barbeau and Guillette, 2007). However, plasma steroid concentrations of testosterone (T) and estrogen (E2) in nitrate-exposed *X. laevis* were unaffected.

An extensive body of work represented by the above studies, continues in the area of nitrate exposure in amphibians and how or if they correlate to the current decline and changes in amphibian survival. My thesis research uses chronic exposure and takes account of total body condition at multiple stages to evaluate progress at time to metamorph, at metamorphosis, and two months post metamorph. Evaluating total body condition is important and represents a holistic approach to evaluating the effects of nitrate and looking at the total exposure time as not just a brief instance looking at one specific attribute (effect) but on multiple endpoints so that the most data for chronic exposure could be gathered.

1.2 The Global Nitrogen Cycle with Emphasis on Nitrate

The global nitrogen (N) cycle serves critical needs in ecosystem function. At the organism level, nitrogen is needed for synthesizing a diverse number of compounds (e.g., amino acids, proteins, vitamins, and DNA) and, at the community level, nitrogen availability is frequently a limiting factor in species diversity (Bernhard, 2012). Paradoxically, this essential nutrient becomes toxic to both organisms and ecosystems in high concentrations of certain nitrogen forms (Bernhard, 2012). High concentrations of ammonia in aquatic systems are directly toxic to living organisms and continued high levels of nitrogen added to aquatic ecosystems can lead to eutrophication and algal blooms followed by oxygen deprivation (anoxic) and death of organisms (fish kills). Nitrogen may occur as a variety of mobile reactive and non-reactive forms of organic or non- organic origins in the global nitrogen cycle.

Nitrate ($NO3^-$) (CASRN: 14797-55-8) is the most stable and abundant of the nitrogen ions and is especially concentrated in aquatic ecosystems adjacent to agriculture settings that are sources of manure and fertilizer run-off. In general, nitrate is the form that is taken up directly from the soil/water by green plants for biosynthetic activity. The primary forms of nitrogen in soil include ammonia (ammonium ion), nitrate, and nitrite. Processes that drive the formation of nitrogen in soil include natural processes such as nitrogen-fixation, microbial nitrification, ammonification (an outcome of microbial decomposition), and anaerobic denitrification as well as anthropogenic inputs of sewage, agricultural/industrial waste, and fertilizers. Nitrate is highly soluble in water (USEPA Nitrate in Ground Water, 2008) when added to soil/sediment structures and may permeate lower into ground water. Nitrate loss occurs through three biological and/or chemical processes: leaching, volatilization, and microbial denitrification (Hofman and Cleemput, 2004).

Nitrate in an aquatic environment will accumulate or occur in four forms, listed from most to least toxic: ammonium ions, ammonia, nitrite, and nitrate (Rouse et al., 1999). Contamination of aquatic ecosystems by anthropogenic sources of nitrate, primarily through agriculture and storm water, is an increasing global concern with respect to the health of humans and wildlife. Contamination of local watersheds has increased due to intense nitrogen fertilizer use that has exhibited a more than 20-fold increase since the 1960's in the United States (Rouse et al., 1999). Global application rate of nitrogen for 1991 was estimated at 72 million tons (International Fertilizer Association, 2008). The U.S. reported releases of nitrate compounds that

totaled over 291 million pounds in 2005 (U.S. EPA, 2006). Total global ammonium nitrate production for 2007 was 14,596,000 tons and an increase of 3.6% from the previous year. Globally, ammonia (NH_3) produced during the same year was 126,872 million tons (International Fertilizer Association, 2008). Urea production that year was 3.9% higher than the previous year for a total world production level of 66,302,000 tons.

Each year, the human population consumes approximately 25 million tons of protein nitrogen. With the expanding world population and increases in resource extraction, the amount consumed is expected to reach 40-45 million tons by 2050 (Guillette and Edwards, 2005; Jenkinson, 2001). Humans fix by industrial processing 160 million tons of nitrogen per year and of that amount, 83 million tons is used in agricultural fertilizer (Guillette and Edwards, 2005).

Three primary types of inorganic nitrogen fertilizers are used extensively in agriculture: ammonium ($NH4^{+}$), nitrate ($NO3^{-}$), and urea ($CO(NH2)2$). Fertilizers are applied in liquid and solid form for agriculture. Nitrate fertilizers, which have been the focus of many toxicology studies since the 1970's, include sodium nitrate, potassium nitrate, and/or ammonium nitrate forms. All of these are toxic to wildlife although some nitrate forms are more directly toxic than others. Impurities in fertilizers include cadmium, lead, and mercury (USEPA, 1999).

Nitrate contamination levels are determined by the United States Environmental Protection Agency (USEPA) to achieve maximum containment levels (MCL). These levels were established through the Clean Drinking Water Act in Congress in 1974. Nitrate and nitrite MCL values, based on standardized EPA lethality test protocols, are 10 mg/L and 1mg/L, respectively. The MCL is not based on sub-chronic (non-lethal) exposure.

1.3 Mode of Action and Effects of Nitrate Exposure

Nitrate is necessary at low concentrations for growth and development but becomes toxic at higher concentrations or in forms that are not easily metabolized. Acute oral exposure to sodium nitrate in animals such as mice, rats, and rabbits produces an LD50 (lethal dose at which 50% lethality occurs) at 1600-9000 mg of sodium nitrate per kilogram of body weight. Nitrite produces an LD50 of 85-220 mg of sodium nitrite per kilogram of body weight in similar animals (World Health Organization, 2007). At levels below its MCL, nitrate is considered safe for wildlife and human adults. Although not usually present in lethal concentrations, recent research discussed below indicates that nitrate concentrations at or below

drinking-water standards may have deleterious chronic effects on wildlife and humans. The No-observable-adverse-effect-level (NOAEL) is 10 mg nitrate-nitrogen/L (1.6 mg/kg/day) and the Lowest-observable-adverse-effect-level (LOAEL) is 11-20 mg nitrate-nitrogen/L (1.8-2.3 mg/kg/day) (Integrated Risk Information System, EPA Nitrate (CASRN 14797-55-8)).

Nitrate poisoning occurs through exposure routes that include ingestion and absorption through permeable skin (Ellis, Myazdanpara, Makela et al. 1998). After ingestion, nitrate ($NO3^-$) is converted to nitrite ($NO2^-$) in the small intestine, absorbed into the blood stream (nitrite is oxidized to nitrate in the blood), and then metabolized via nitric oxide. Nitric oxide converts hemoglobin (Hb) to methemoglobin (metHb) through NADPH dependent reductase (Bruning-Faun and Kaleene, 1993). The Fe^{2+} present in the heme group is oxidized to its Fe^{3+} form that does not bind oxygen and the remaining nitrite binds firmly to the oxidized heme (World Health Organization, 2007). Consequently, methemoglobin reduces the ability of the blood to carry oxygen because the affinity of the molecule is greater for nitrite than oxygen. High pH in the small intestine enhances the conversion of ingested nitrate to nitrite. Approximately 5% of the ingested nitrate is converted to nitrite. The major part of the ingested nitrate is eventually excreted in urine as nitrate, ammonia or urea along with some possible fecal excretion (WHO, 2007). After ingestion, nitrate can be converted to N-nitroso compounds and nitric oxide (NO) which is then reduced to nitrous oxide (N20).

Nitrate/nitrite intoxication occurs through systemic accumulation of these compounds and is correlated with age-dependent factors in exposure toxicity. Acute toxicity may exert deleterious effects such as rapid pulse, dyspnea, muscle tremors, weakness, vomiting, unstable gait, cyanosis, weight loss, vitamin A deficiency (a shortage of vitamin A negatively influences thyroxine which is critical to regulating time to metamorphosis in amphibians) and, decreased function of the thyroid gland (nitrate competitively inhibits iodine uptake), decreased fertility, and abortion (WHO, 2007). All such signs and symptoms of nitrate/nitrite toxicity are related to the effects of methemoglobin (the inability to bind oxygen, resulting in cyanosis and anoxia). The nitrite drinking water standard of 3 mg/L is based on infant methemoglobinaemia levels of 0.4 to more than 200 mg/kg of body weight (WHO, 2007). Short-term acute effects in humans include shortness of breath and blueness of the skin caused by inhibition of the blood's oxygen-

carrying capacity (USEPA, 2008). Long-term effects of exposure include diuresis, increased starchy deposits, and hemorrhaging of the spleen (USEPA, 2008).

1.4 Fluctuating Asymmetry

While perfectly symmetrical organisms should exhibit no measureable variation in right and left side traits, such as bone length or number of rays in a pelvic fin, some degree of asymmetry in trait development may occur due to different reasons. Fluctuating Asymmetry (FA) is a measure that may reflect developmental stability or instability of an organism's physical traits. Measures of FA may be confounded by effects of either antisymmetry (AA) or directional asymmetry (DA) and these contributions to asymmetry do not reflect developmental stability. Developmental stability (DS) is the degree to which an organism maintains homeostasis during early developmental stages and is not influenced by disrupting factors. To a limited extent, organisms are developmentally buffered and can suffer from some susceptibility to environmental and genetic stress. Asymmetry in right and left side character traits in an organism may occur due to FA, AA and DA, three distinct types of asymmetry. Antisymmetry (AA) is a bimodal distribution with no consistent pattern of variation among individuals (Palmer, 1996; Van Valen, 1962). Antisymmetry is graphically represented by a broad peaked or bimodal distribution of Right – Left side differences about a mean of zero. For example, male fiddler crabs have a single oversized claw occurring with approximately equal frequency on either the right or left sides in nearly all species. Directional asymmetry (DA) is a pattern of bilateral variation that is consistent in the side displaying the trait (bias of a character towards greater development on one side of the body than the other) where statistically significant differences exist between sides. In a graphical frequency distribution of R – L side measures, DA is shown as skewed from the mean. Fluctuating asymmetry (FA) is the random deviation from perfect bilateral symmetry (Van Valen, 1962). This measure is also defined as a morphological feature that is manifested when an organism is unable to undergo identical development of an otherwise bilaterally symmetric trait on both sides of the body (Moller, 1993). Fluctuating Asymmetry represents an epigenetic measure of developmental homeostasis and the sensitivity of developmental stability or stress (Moller, 1993).

Palmer and Strobeck (1996) developed the statistical technique for using FA as a tool to monitor environmental stress in an organism during developmental periods in 1986 and refined

it in 1996. Their proposed estimate for FA focused on a normal distribution of R – L differences whose mean is zero.

$$FA_i = |(R_i - L_i)| / [(R_i + L_i)/2]$$

R_i = trait on the right side

L_i = trait on the left side

Average FA: $(AVFA) = (\sum FA_i)$ / total # of traits

Palmer and Strobeck proposed that the measurement of FA may reflect an organism's degree of genetic heterozygosity. Some studies reported a positive correlation between FA and amount of antisymmetry in traits with an increase in genome wide homozygosity. Relevant to studies of environment impact, FA was proposed as a measure that may reflect environmental stress such as pesticide exposure encountered by an organism during development. A number of ecotoxicologists therefore proposed FA as a valid bioindicator of the organism's previous exposure to environmental contaminants as well as future ability to withstand continued environmental perturbation.

1.5 Purpose of the Study

Nitrate applications in agriculture are utilized globally, increasing the importance of evaluating their effects on aquatic systems. The majority of nitrate toxicity investigations used acute or short term monitoring methods. Use of a chronic testing method, as proposed in this study, may be advantageous when looking for long-term sub-lethal effects caused by lower, environmentally realistic concentrations. Numerous ecotoxicological studies utilize anuran amphibians after larvae gills are internalized, referencing Gosner stages 25 to 32 and therefore monitor later developmental stages. This study begins at an earlier stage in development and tests the compound for sensitivity to a long period of exposure throughout development. The continued or discontinued chronic nitrate exposure after metamorphosis provides a unique perspective on developmental periods and growth. Use of sodium nitrate produces a single chemical stressor to illicit a dose-response for tissue examination. The use of a single chemical provides a base-line analysis for future study of mixture studies. Other studies that used ammonium nitrate may have experienced a confounding factor since the ammonium portion of the compound is a known aquatic toxicant. Water quality measurements taken during water cycles monitor other potential sources of nitrate such as food and waste.

In this study, toxicological effects evaluated as important evidence for nitrate as an endocrine disrupting chemical include intersex gonads (ovotesis), disrupted steroidogenesis, disrupted spermatogenesis, and altered gonadal development. Other indications of toxicological effects of nitrate include lethality and external malformation.

In this study, I examined how long term, sub-lethal exposure to environmentally relevant concentrations of aquatic nitrate influences tissue level biomarkers (hematocrit, liver and gonad histology, and organism symmetry) in developing *Xenopus laevis*. The purpose of this study was to examine the relationships between nitrate chemical exposures and physiological function in the model species. Results contribute to the growing list of potential toxic effects that sub-lethal nitrate concentrations pose to amphibians. This research provides evidence for the use of tissue- level histological measures, hematocrit, and FA as indicators of nitrate exposure. It is anticipated that the results from this study will contribute to a baseline of research for nitrate chronic exposure in amphibians by establishing end points for biomarkers, hematocrit, and structural symmetry. Future combination studies will be able to observe possible synergistic or negative effects of combinations with other toxicants. The null hypothesis for this experiment is: the nitrate treatments will have no effect on any of the bioindicators selected.

CHAPTER 2

METHODS AND MATERIALS

The study was designed to be multifactorial. The first segment of the research involved the breeding and rearing tadpoles in five concentrations (treatment groups) of nitrate, until metamorphosis at which time measures were taken of the froglets. The second segment of this study was to divide of each treatment group into sub-groups a) those that continued receiving the nitrate dosed or b) discontinued receiving the treatment, until two months post metamorphosis. At two months post metamorphosis, all treatments ceased and final measurements were taken. After animal preservation, FA measures were taken and tissues were removed for slide preparation. A flow diagram of the study design is presented in Appendix B.

2.1 Study Species; *Xenopus laevis* and Over View of Method.

Dr Brown and the lab have a history of using *Xenopus laevis* and extensive experience in the breeding and experimental use of *Xenopus (X.) laevis*. This species is also used in many other labs and so is presented in a wide variety of relevant literature for studies of nitrate and endocrine disruption, tissue histology and response to environmental stress. Further, *Xenopus leavis* is relevant because its entire life cycle is aquatic. The genus *Xenopus*, as adult frogs, are not ureotelic (excrete ammonia) unlike the genus *Rana*. This is advantageous because ammonia would be a confounding factor for this study. One of the problematic aspects of their use is that they are considered invasive and not native to North America.

2.2 Animal Breeding and Larval Care.

Four breeding pairs of adult *Xenopus laevis* were obtained from Xenopus I, Inc. (Dexter, MI, USA). Animal cultures from Xenopus I are regularly replenished with individuals from wild populations to avoid inbred cultures. Upon arrival at the laboratory, the body condition of the adults was observed and the males and females were separated and housed in 85L polypropylene tubs (Sterilite Corporation) filled approximately with one-third Moderately Hard Water (MHW) (Appendix A). Animals were acclimated/observed for two weeks.

Light/Dark Cycles matched ambient light from north facing windows and were supplemented with fluorescent lights. Temperature in the laboratory varied between 20°C and 22.2°C. Adults were fed ad- libitum using adult mix food from Xenopus I; adult females were fed every two days and males every three days. A complete water change and tank cleaning using hot water and deionized water rinse (described below) was done the day after feeding.

2.3 Breeding

The breeding pairs were selected at random. Pairs were housed in breeding tanks with 1cm grid plastic flooring (mesh panels) to protect fertilized eggs from the adult claws. Breeding was induced with a single injection of human chorionic gonadotropin (hCG) (American Pharmaceutical Partners Inc. Schaumburg, IL), 1000 I.U., into the dorsal lymph sac of each male and female. After amplexus, at approximately 24 to 48 hours and subsequent gamete deposition, adults were removed from the breeding tanks. Immediately thereafter, air stones were placed in the tanks and additional MHW was added to increase water depth until hatching was observed.

2.4 Larval Husbandry

Larvae were reared in tanks with stone aeration. A water temperature of approximately 22°C was maintained to encourage development. Hatching occurred approximately 96 hours following fertilization. At the time of hatching, feedings and tank maintenance regimes, such as removal of debris and unfertilized eggs, were implemented. Tadpoles at the free-swimming stage were collected daily and placed in a 38 liter (10 gallon) glass aquarium by clutch and aerated until random selection for nitrate treatments.

Metamorphosis is regulated both by the external (environment) and internal (hormonal) processes. Factors that regulate the rate of metamorphosis include temperature (Hayes et al., 1993), food levels and predator pressure (Werner 1986), tadpole densities (overcrowding) (Semlitsch, 1988), and water levels (Denver et al., 1997). Hormonal control at metamorphosis is dependent on the following thyroid hormones: Thyroxine, T4; triiodothyronine, T3; and iodinated forms of the amino acid tyrosine. Thyroid hormones stimulate jaw and head transformations during metamorphosis (Hanken and Summers, 1989) and skull ossification (Hanken and Hall, 1988). The

skull measures were taken for FA analysis but ultimately discarded due to low confidence in the data collected.

2.5 Experimental Design.

Twenty-five tadpoles, randomly selected from each clutch, were allocated into each of the replicate tanks for a total of 500 tadpoles in the entire experiment. After the initial introduction of tadpoles into the experiment, non-surviving tadpoles, the following three days, were replaced to maintain density. During early development stages, the tadpoles were housed in 1.5 L mason jars that had been autoclaved and stone aerated. Moderate hard water was prepared in seventy-two liter quantities. Concentrations of nitrate (NaNO3) from Sigma Chemical in St. Louis, MO, USA were determined by the percent of elemental nitrate present and then measured per volume of water. Concentrations were chosen based on literature research, previous studies in the Brown laboratory, and environmental health standards to create a range that was lower than drinking water standards but environmentally/agriculturally relevant. The concentrations used are not lethal in this species as determined by previous research conducted in Brown laboratory (Spence and Brown, 2002).

Nitrate concentrations (mg/L) were: 0, 10, 50, 100, and 200, with four replicate tanks per treatment level (nitrate concentration). After significant growth to Gossner stage 21, the tadpoles were transferred back into 10 gallon (37.85 liter) glass aquariums with 6L of MHW water. The housing conditions for post-metamorphic froglets were the same as for pre-metamorphic tadpoles, except that additional water and chemical was added to increase total tank volume for the larger animals. Tanks were tilted to allow for an increase in water depth and a white background paper was applied under the aquaria to facilitate observations.

2.6 Water Changes, Tank Cleaning, and Feedings

Complete water changes and chemical renewal occurred every 72 to 96 hours. Tanks were cleaned by gently pouring off water and removing the animals to a holding tank. The tanks were cleaned with hot tap water, wiped down with an unbleached paper towel to remove buildup, and re-rinsed with deionized water. An appropriate sodium nitrate concentration was added directly to the tank, mixed with allocated water, and the animals returned. Xenopus

tadpole mash was prepared for feedings. A mixture of tank water and 20-15 grams of mash was mixed within a jar and added until water was clouded with feed.

2.7 Water Quality Standards

Water quality measures were made for five water renewal cycles (3-4 days of each nitrate treatment and control) using a HACH test kit and DR-890 colorimeter. Analyses were performed daily (24 hours) to measure available nitrate (HACH Method 8039), nitrite (HACH Method 8153), total nitrogen (HACH Method 10071), and nitrogen ammonia (HACH Method 8155). The purpose was to document the stability of the nitrate added to the water system and monitor any accumulation of ammonia and nitrite.

2.8 Metamorphic Data Collection

Metamorphosis was determined by the development of hind legs, appearance of legs, and complete absorption of the tail (G stage 46). Metamorph collection and measurements occurred from April 7, 2008 until May 15. As metamorphs emerged from any replicate tank within a treatment, they were removed daily and measured for snout-vent length (SVL) in millimeters using a caliper and dry weight in grams. Metamorphs were transferred to new tank treatments. Twenty-five froglets from each treatment were continued at the dose of sodium nitrate experienced as tadpoles and another twenty-five froglets from the same treatment group were housed in control MHW only. Nine treatments in total were maintained from metamorphosis to two months post metamorphosis. These nine treatments involved the five nitrate treatments that the tadpoles were reared in crossed with whether or not the frogs continued to be exposed to that nitrate concentration after metamorphosis (Table 1).

Table 1: Treatment Exposure Levels for Nitrogen in Nitrate

Tadpole Exposure Treatment Level (mg/L)	Post Metamorphosis to 2 Months Post Metamorphosis (pre metamorph, post metamorph)	
	Continued Treatment	Discontinued Treatment
0	0, 0	0, 0
10	10, 10	10, 0
50	50, 50	50, 0
100	100, 100	100, 0
200	200, 200	200, 0

2.9 Post Metamorphic Data Collection

At approximately two months post metamorphosis, a final wet weight and snout-vent length was obtained for 179 animals. Focal animal measures were taken from July 9, 2008 until July 28, 2008. Individual animals were assigned an identification label and number indicating both pre-metamorphic and post-metamorphic treatment. Animals (n = 168) were individually anesthetized using 1.5 g/L MS-222 (Sigma Chemical, St. Louis, MO, USA) and dissected ventrally to perform a heart puncture from which blood was drawn with a heparinized capillary tube. The tube was put into a centrifuge to separate blood platelets from the plasma fraction. A reading of the percent of red blood cells to total blood volume (hematocrit) was recorded.

2.10 Preservation

Following heart puncture, animals were euthanized in MS-222 and their thoracic cavity was opened from sternum to vent to freely expose internal organs to fixative. Bouin’s fixative was applied for two days followed by 10% formalin. Finally, animals were rinsed and then stored in 70% ethanol using 1 L glass jars with identification tags attached to a hind leg.

2.11 Fluctuating Asymmetry

Before dissection work to prepare reproductive and liver tissue for histology analysis, the animals were taken to Indiana University School of Medicine in Terre Haute. Under the direction of Dr. Michael Lannoo and his staff, the animals were radiographed using a Fixatron

radiograph machine with a Cabinet X-Ray System (ID: 4385513, S/N 43855B; calibration date: 01/04/2007). Animals were arranged in sequence of exposure level and according to identifying tag. Tags were removed temporarily during the procedure. An exposure time of three minutes and ten seconds was used to produce the clearest resolution. The films were developed and loaded in a dark room. Within the radiograph envelope there was a plastic cover, film, plastic liner, and then lead that reduces radiation bounce-back and improves the image.

The films were transformed into digital files for analysis. A Nikon Cool Pix 8700, 8 megapixel, Zoom Nikon ED 8.9-71.2mm 1: 2.8-4.2, with the following setting was used: macro digital, black and white, auto focus, three second exposure time, and maximum zoom. Radiographs were shot on a light table, with ambient light provided from compact white light bulbs, and fluorescent light exclusion. A scale (cm) was used to maintain accurate measurements when digital files were imported into Image J (National Institute of Health, Image Processing and Analysis in Java, Image J, 1.410/Java 1.6.0). To prevent any measurement bias due to knowledge of specific treatment level, a number was randomly assigned to each frog and tagged on the film. This random number was then correlated to the actual tracking number assigned to the frog after measurements were made. IMAGE J was selected in an effort to achieve a low measurement error (ME) and maintain bone articulation. Two-hundred and thirty frogs were digitally surveyed twice with the software for twenty-eight different anatomical traits.

Specific bone measurements used for FA analysis were determined after thorough examination of digital images. Measures of femur width and length, lordosis angle of the spinal cord, and several measures for the skull were included. For measurement accuracy, each individual measure was taken twice and an average value used. The two measures were conducted in separate runs to avoid bias that might occur from having taken the same measurement consecutively. See appendix C for measurement descriptions.

Figure 1: X-ray image saved to digital file.

2.12 Dissection Techniques and Histology

During dissection, animals were determined as male or female by observing gonadal structures. To analyze the histological data, five females and five males were selected from each treatment group for ninety animals in total. Surgical grade micro-scissors and fine tip forceps were used. The animals were removed from storage and the tag removed for a wet weight measurement in 70% ethanol. The ventral opening used for preservation was used to access internal organs. Under a dissecting microscope, the heart was removed and thereby both lobes of the liver were accessed. The liver was then removed and both lobes weighed together in 70% ethanol. The liver somatic index (LSI) was calculated to determine the physiological status of the liver. LSI was calculated using the wet weight of the liver (mg) divided by the total body

weight of the animal (prior to dissection) and multiplied by 100. The animal's body weight before organ removal was determined by immersion in a 25 ml glass beaker partially filled with 70% ethanol and tared to 0g.

The stomach and intestines were removed as a whole with care taken not to disturb underlying fat bodies. Fat bodies were then removed and the reproductive organs detached from the kidneys. Left and right reproductive organs were weighed together (mg) in 70% ethanol as described above. Ovarian tissue was identified as a multi-lobed structure extending the length of the kidney and having melanin granules. Testes were identified as dense tissue attached to the kidneys with connective tissue and an attachment to the fat body on their proximal end. The gonad somatic index (GSI) was used as a ratio of the gonad weight (GW) divided by the total body weight (TW) and expressed as a percentage of total body weight. Post dissection, organs were stored in 5 ml scintillation vials with 70% ethanol and tagged for shipment to Histo-Scientific Research Laboratories in Mt. Jackson, VA. Animals were also stored in 5 ml scintillation vials with 70% ethanol. Organs were embedded in paraffin and step sectioned with a microtome and sections fixed to glass slides. Five sections per organ per slide were prepared by Histo-Scientific Research. Slides were stained with standard Hematoxylin and Eosin (H&E) stain. When the slides were returned, tissue evaluations were conducted using digital images and analyzed using the National Institute of Health, Image Processing and Analysis in Java, IMAGE J, 1.410/Java 1.6.0_07. The Color Atlas of *Xenopus laevis* Histology (Wiechmann and Wirsig-Wiechmann, 2003) was referenced for normal histological characteristics of tissue. Tissue evaluation was paired with a Histology Scoring Index (HAI) for the liver and gonads.

The HAI was used to evaluate periportal, interlobular, and portal inflammation, as well as signs of fibrosis. Gonad measures were included for evaluating gross morphology of the ovary and testis. Oocyte structure or early stage development, sperm and duct development, abnormalities, and condition were recorded. During dissection, any anomalous structures were identified and photographed using a dissecting microscope camera. Those structures were removed, some along with the kidneys, and received individual instructions for slide preparation.

2.13 Liver Histology

The main functions of Kupffer cells (macrophages) are to remove apoptotic cells from the liver, aid in metabolic functions, homeostasis and defense by regulation of inflammatory responses within the liver (Naito et al, 2004). The distribution of Kupffer cells may indicate a response to stress. A sub-population of ten representative animals from each treatment group was selected for slide preparation. Each slide contained five sections from the step sectioning of the liver. To determine the density of Kupffer cells, a random section of the slide was selected as the field of view at 40x magnification. Images were recorded digitally and Image J software was used to open them for counting. An average of five sections was analyzed per animal and counts were evaluated using Statistical Analysis System (SAS), PC Version 9.1. After the survey and analysis of the data, it was determined that the data was not robust and that the development of descriptive statistics would be more complete. What was perceived to be "damage" was assessed in a large number of the slides prepared. The determination of "damage" was made when large, fractures or tares in the tissue were observed or when the periphery of the liver section was greatly disturbed.

2.14 Statistical Analysis

Statistical analysis was conducted using SAS. Data were treated assuming a normal distribution, utilizing ANOVA, General Linear Models, and Tukey's Studentized Range (HSD) Test. Statistical significance was set at a $p \leq 0.05$ level. Confidence intervals were specified as two standard deviations from the mean.

Water quality analysis was conducted for each treatment group and each day of the water cycle. Five treatment levels were analyzed (0, 10, 50, 100, 200 mg/L). One way ANOVA was used among nitrate treatments (days pooled). Pooled days indicate one water 'change' treatment cycle and encompassed four days. The variables measured among pooled days were: nitrate, nitrite, and nitrogen ammonia. An ANOVA for water treatments among days (1-4) was used to indicate temporal variability between the following variables: nitrate, nitrite, ammonia, days.

Second, the pooled animal metamorphic data was analyzed to compare the rate, size (snout-vent length), weight, and observable fitness between continued nitrate treatments and discontinued treatments compared to the control. Because animal data was pooled among replicate tanks into single tanks for each treatment, individual metamorphic data is not available. A one-way ANOVA test (Ho: $X_c = X_{10} = X_{50} = X_{100} = X_{200}$), was used.

The raw data included 479 observations among five nitrate treatment groups (0, 10, 50, 100, 200 mg/l) compiled into SAS. The mean values, UNIVARIATE procedure, and GLM with Tukey's Studentized Range were determined for the following variables: snout-vent length, weight, days to metamorphosis, and body index.

Post-metamorphic data was analyzed in a similar method. Analysis was performed between continued and discontinued nitrate within metamorphic nitrate treatment levels (e.g., 50/50 and 50/0 animals grouped) between known animal sex (smaller sub-population data set) within a nitrate treatment and with sexes pooled. A one-way ANOVA was performed to analyze animal focal measures (Ho: $X_c = X_{10} = X_{50} = X_{100} = X_{200}$). Individual data for each frog measured were used and compiled with the hematocrit data.

Body condition was determined as the percent change in body condition from metamorphosis to two months post metamorphosis. The calculation was as follows: percent change equals body condition two months post metamorphosis minus body condition at metamorphosis divided by the post metamorphosis body condition multiplied by 100. Hematocrit data used the mean value as compared to weight, size, and treatment level.

Histology data analyzed gross examination of the liver (LSI) and gonadal (GSI) structure and condition indices. The liver somatic index was calculated as the liver weight divided by the body weight, and then multiplied by 100. The gonad somatic index was calculated as the gonad tissue divided by the wet body weight, and then multiplied by 100. The frequency and degree of abnormalities per treatment were noted. Somatic indexes were compared between treatments. Average gonad tissue weight per animal weight was also calculated.

The significance of the liver Kupffer cell density between treatments was determined by a non- parametric one-way ANOVA among pooled treatment groups. A general linear model (GLM) was used to separate the groups using a Tukey's test. The Tukey's Studentized Range (HSD) test used a comparison of significance at the 0.05 level. The test controls the Type I experiment-wise error rate. A Bartlett's Test for Homogeneity of cell variance was used to determine if and where the differences in treatments occurred.

Incidence of 'damage' was noted per animal. Damage was identified as abnormal appearance of the liver structure such as inclusions or large spaces and possible damage due to tissue preparation for slide sectioning. A chi-square test was used to evaluate significant associations between occurrences of damage and treatments.

Descriptive statistics were used for right and left (R-L) differences for morphological characters used in measurement of FA. Deviations from bilateral symmetry, directional asymmetry (DA), anti-symmetry (AA), mean (Y), skewness (g1), and kurtosis (g2) estimates for the frequency distribution of right and left side difference (R-L) were included for each character. The methods and statistical approach reference a previous fluctuating asymmetry method conducted in the lab by Allenbach et al (1999). Repeat measures were conducted to estimate measurement error.

Twenty eight measures were performed twice for each frog and the image was recorded digitally. Characteristics were checked for frequency distribution of the left minus right values and then the skewness and kurtosis to access the anti-symmetry but none were indicated. Then the mean was evaluated for zero and determined if there were any outliers. It was expected that the left-right values should have a normal distribution, indicating that the skewness and kurtosis were equal to zero and also that the mean of the left-right values should also be equal to zero.

CHAPTER 3

RESULTS

3.1 Water Quality Data Analysis: Nitrate

Mean nitrate values differed significantly among treatments (Table 2; $F = 48.34$, $p < 0.0001$, d.f. = 4, 84). Except for treatments 0 mg/L and 10 mg/L, all treatments differed significantly from each other in the expected manner (i.e., increasing means with treatment levels). The expected rejection of the null hypothesis (Ho: $NO3_{0} = NO3_{10} = NO3_{50} = NO3_{100} = NO3_{200}$) indicates an appropriate dose was administered to each treatment group. The rate of dissociation (sodium nitrate + water → sodium hydroxide and nitric acid) and the fraction of nitrogen (approximately 73%) in the nitrate was considered when the exposure volumes were developed (Appendix A). 1.37 g of $NaNO_3$ is equivalent to 1 g of NO_3. The nitrate found in the source water was not considered when the dosing scheme was developed. Other possible sources of nitrate, such as the animal feed, were also not considered.

Mean nitrate values differed among days in the control treatment (Table 3; $F = 17.73$, $p < 0.0001$, d.f. = 3, 14) and 10 mg/L treatment ($F = 10.94$, $p < 0.0006$, d.f = 3, 14), particularly between days 1 and 4 and days 2 and 4. Nitrate consistently increased with each day following water changes. Nitrate levels in the other treatment groups did not differ among days as in the control because the administered concentrations were much higher than background nitrate levels.

3.2 Water Quality Data Analysis: Nitrite

No significant difference in mean nitrite was found among treatment levels (0, 10, 50, 100, and 200 mg/L). No temporal variability was indicated among days within any treatment group. Nitrite did not accumulate above published water quality standards.

3.3 Water Quality Data Analysis: Nitrogen Ammonia

No significant difference (Figure 3) was found among treatment levels (0, 10, 50, 100, 200 mg/L) in ammonia concentrations. No temporal variation among treatment days occurred. Nitrogen ammonia did not accumulate above published water quality standards.

Table 2: Mean water quality values during water renewal periods for each treatment group. Data given as mean and 2 standard deviations in parentheses.

Treatment Level (mg/L)	NO3 (mg/L)	NO2 (mg/L)	NH3 (mg/L)
0	3.14 (1.18)	1.55 (0.64)	0.09 (0.02)
10	5.2 (1.36)	2.44 (1.02)	0.08 (0.02)
50	11.01 (1.36)	2.38 (0.94)	0.09 (0.06)
100	17.77 (3.44)	3.55 (1.94)	0.08 (0.02)
200	27.04 (4.82)	3.00 (1.66)	0.07 (0.02)

Table 3: Variation of water quality between days within a water renewal period. Means with two standard deviations in parenthesis are shown for NO3, NO2, and NH3. Results for Tukey's Studentized Range (HSD) test are given as letters; if two means do not share the same letter, they are significantly different.

Treatment Level (mg/L)	Day of water cycle	NO3 (mg/L)	NO2 (mg/L)	NH3 (mg/L)
0	1	0.68 (0.21) A	2.00 (0.89) A	0.06 (0.01) A
	2	2.00 (0.36) AB	2.20 (0.49) A	0.08 (0.01) A
	3	4.45 (0.41) BC	0.25 (0.25) A	0.14 (0.32) A
	4	6.35 (1.18) BC	1.50 (0.29) A	0.09 (0.04) A
10	1	2.28 (0.37) A	2.40 (1.25) A	0.04 (0.02) A
	2	4.30 (0.38) AB	2.40 (0.93) A	0.07 (0.03) A
	3	6.63 (1.06) BC	2.50 (1.32) A	0.15 (0.03) A
	4	8.55 (1.40) BC	2.50 (0.96) A	0.11 (0.03) A
50	1	8.60 (0.43) A	2.00 (0.89) A	0.03 (0.02) A
	2	11.26 (0.79) A	2.80 (1.20) A	0.07 (0.03) A
	3	12.08 (1.21) A	2.25 (0.63) A	0.12 (0.05) A
	4	12.68 (2.35) A	2.50 (1.19) A	0.21 (0.16) A
100	1	14.24 (1.71) A	2.40 (1.25) A	0.05 (0.02) A
	2	18.10 (4.45) A	4.40 (2.79) A	0.07 (0.03) A
	3	21.05 (4.85) A	2.50 (1.19) A	0.09 (0.04) A
	4	18.50 (2.38) A	5.00 (2.27) A	0.13 (0.04) A
200	1	22.26 (2.77) A	2.20 (1.24) A	0.08 (0.01) A
	2	23.90 (3.11) A	3.20 (1.56) A	0.05 (0.03) A
	3	29.53 (5.13) A	2.75 (1.80) A	0.12 (0.06) A
	4	33.68 (7.36) A	4.00 (2.71) A	0.06 (0.02) A

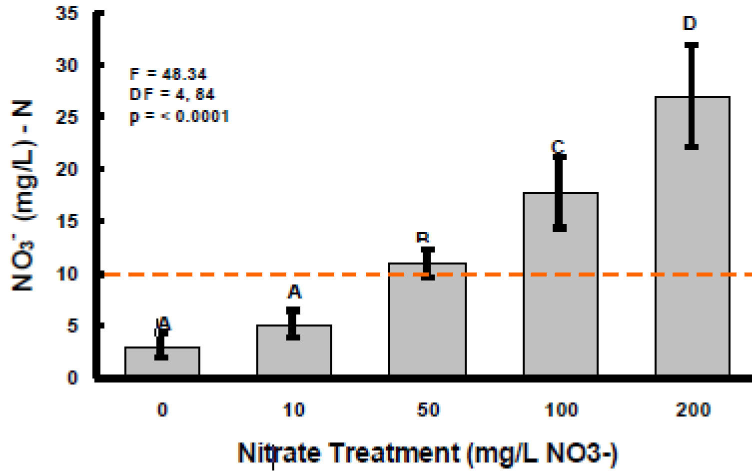

Figure 2: Nitrate levels during water renewal schedule. Brackets represent two standard deviations above and below the mean. No temporal change. Nitrate was dosed as nitrate and not part of nitrogen. Hashed orange line indicates MCL by EPA standard for drinking water.

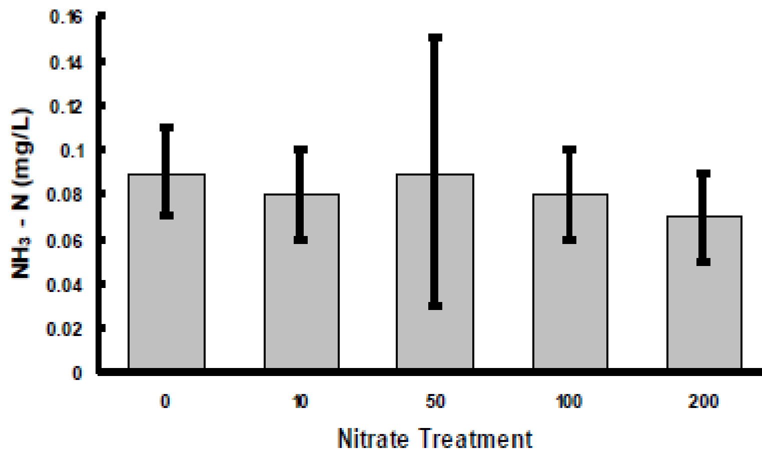

Figure 3: Ammonia levels during water renewal schedule. Brackets represent two standard deviations above and below the mean. No statistical difference between treatment levels.

3.4 Metamorphic Data Analysis

No significant differences among treatments were found for the following variables at metamorphosis: snout-vent length (millimeters), weight (grams), and body condition (weight divided by snout-vent length). A significant difference for days to metamorphosis was indicated between the control and 200 mg/L treatment group (Table 4, Fig. 4, $F = 2.42$, $p = 0.0476$, d.f. =

4, 474). Control animals metamorphosed in fewer days compared to animals in the 200 mg/L treatment.

Table 4: Traits related to metamorphosis among tadpoles of *Xenopus laevis* exposed during the larval period to concentrations of nitrate (0, 10, 50, 100, 200 mg/L). Two standard deviations represented in parenthesis. Results for Tukey's Studentized Range (HSD) test are given as letters; if two means do not share the same letter, they are significantly different.

Treatment Level (mg/L)	Days to metamorphosis	Wet Weight (g)	Snout-vent Length	Body Condition (K)
0	49.29 (1.376) A	0.58 (0.04)	17.38 (0.316)	3.29 (0.864)
10	50.84 (1.538) AB	0.55 (0.038)	16.88 (0.3)	3.19 (0.610)
50	50.76 (1.2) AB	0.54 (0.034)	16.92 (0.324)	3.15 (0.42)
100	49.86 (1.35) AB	0.56 (0.04)	17.19 (0.31)	3.22 (0.478)
200	52.18 (1.508) B	0.58 (0.034)	17.24 (0.3)	3.33 (0.544)

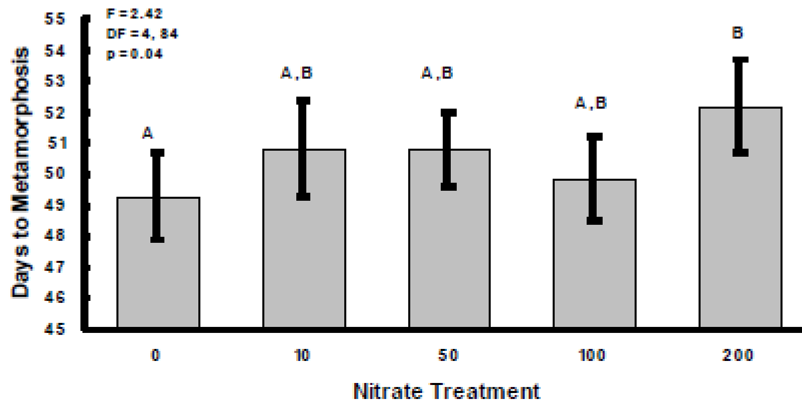

Figure 4: Days to Metamorphosis. ANOVA test showing mean-value histogram showing 2SD at 95% confidence intervals. The p-value was less than 0.05 indicating a significant test. Treatment groups 200 and 0 were statistically significant from each other, therefore rejecting the null hypothesis.

3.5 Two Months Post Metamorphic Data Analysis

Treatments were pooled because no significant difference was found between continued and discontinued treatments. No significant difference among the nine post-metamorphic treatments was found for the following variables at two months post metamorphosis: body condition, hematocrit, liver somatic index, and gonad somatic index. Wet weight of froglets in all groups increased from metamorphosis to two months post

metamorphosis. Froglets were four times larger at two months post metamorphosis compared to their size at metamorphosis (Figure 5, Table 6). However, no significant difference in mean wet weight was noted among treatments. For froglets in all groups, the snout-vent length less than doubled during time after metamorphosis. No difference in mean snout-vent length was noted among treatment groups.

No statistical differences in the separate treatments (if the nitrate treatment was continued or discontinued after metamorphosis) or sexes at two months post metamorphosis were found. Therefore, data was pooled between sexes and within treatments (e.g. 10/10 and 10/0 would be analyzed in the same treatment group, 10). By pooling data, sample sizes were larger and provided a larger statistical base.

Table 5: Percent change in body condition (K) from metamorphosis to two months post metamorphosis.

Treatment Level (mg/L)	**% Change**
0	58.03571
10	60.11004
50	58.76963
100	60.06944
200	59.09091

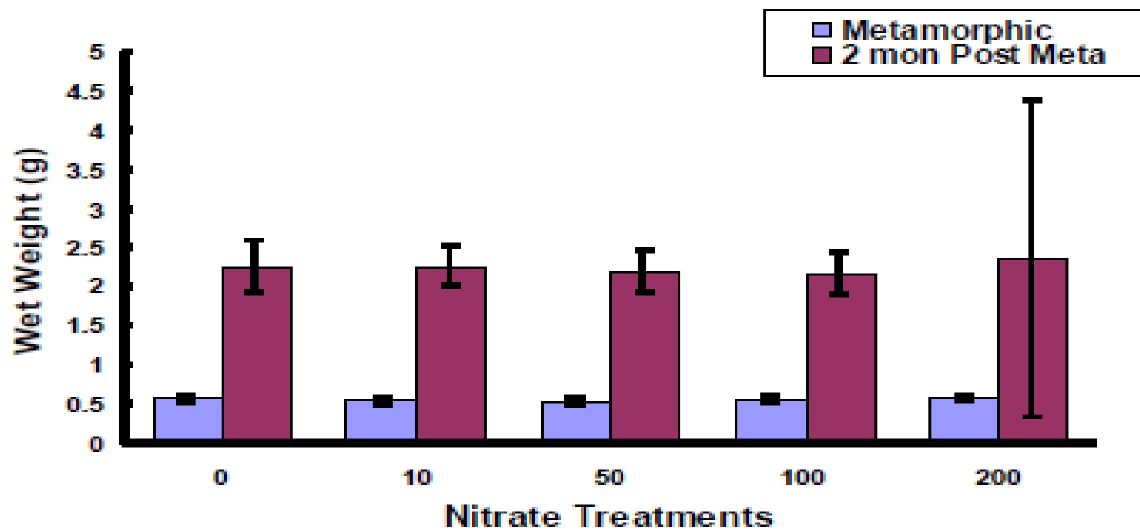

Figure 5: Wet Weight. Increase significantly in size from metamorphosis to two months post metamorphosis. No difference between treatments.

Table 6: Mean values of froglets at 2 months post metamorphosis. For these treatment comparisons, animals exposed to the same premetamorphic nitrate treatment levels were grouped. Data given as mean and 2SD in parentheses.

Treatment Level (mg/L)	Wet Weight (g)	Hematocrit	Body Condition (K)	Snout-vent Length
0	2.257 (0.326)	35.494 (1.680)	7.840 (0.864)	28.397 (1.376)
10	2.260 (0.248)	35.590 (1.746)	7.997 (0.610)	27.66 (1.000)
50	2.193 (0.262)	22.380 (1.680)	7.640 (0.420)	27.53 (0.704)
100	2.170 (0.262)	35.885 (1.480)	8.064 (0.478)	28.38 (0.784)
200	2.370 (2.020)	35.450 (1.352)	8.140 (0.544)	28.51 (0.278)

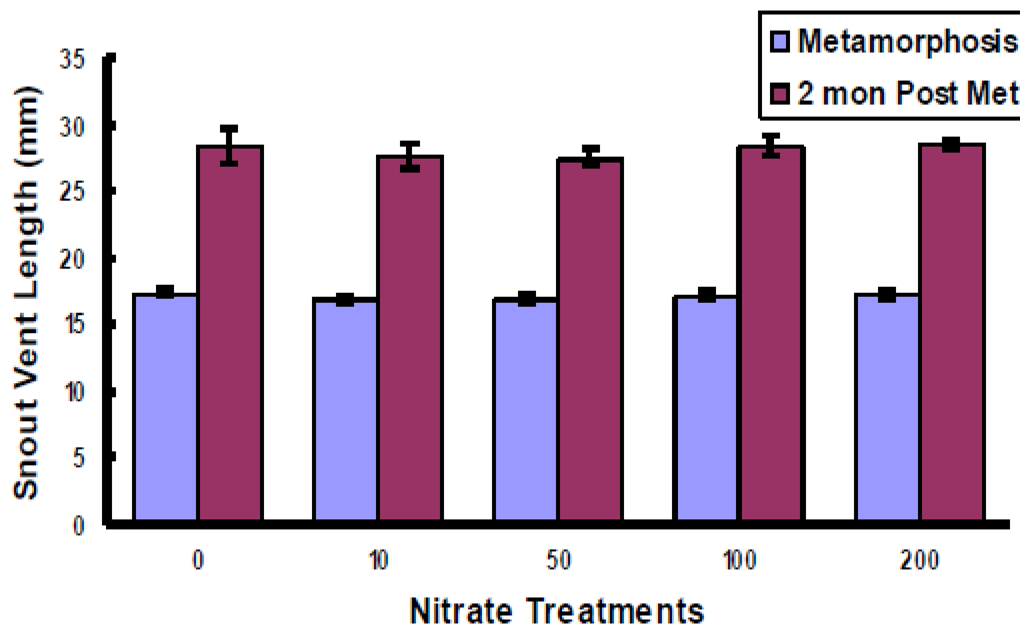

Figure 6: Snout Vent Length (mm) by nitrate treatment group (mg/L). Mean values were not significantly different at metamorphosis or at 2 months post metamorphosis. The length of the animal less than doubled after two months of development.

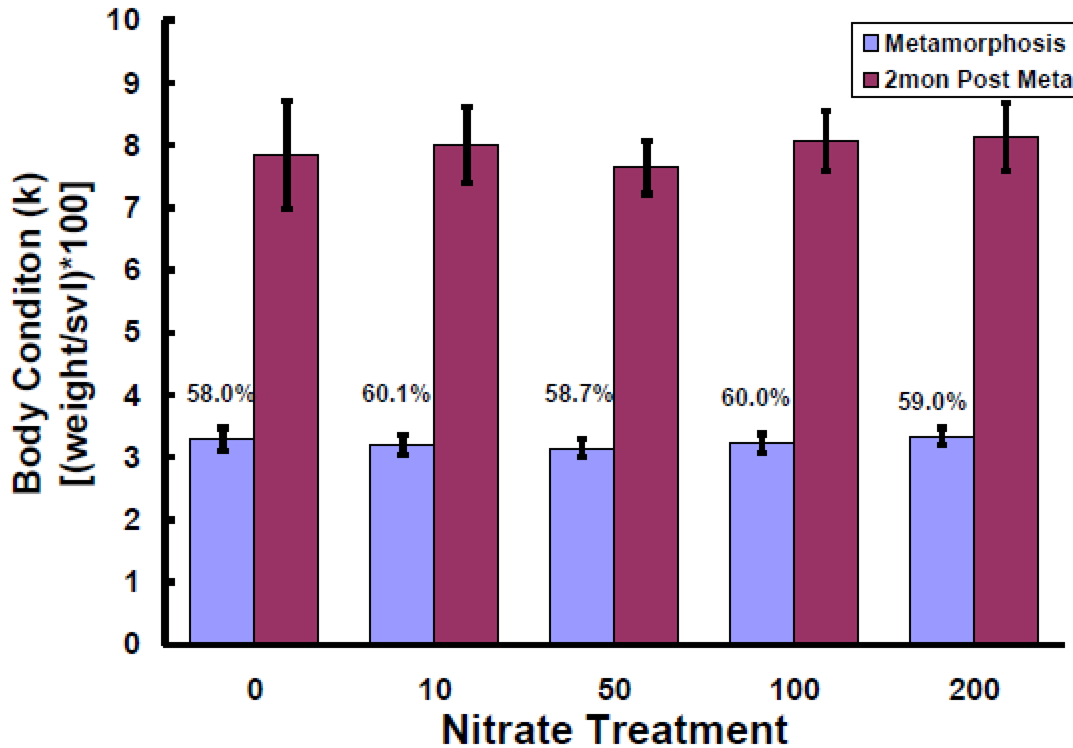

Figure 7: Body condition at metamorphosis and 2 months post metamorphosis. Body condition as a measure of "plumpness". The figure indicates the percent change in body condition from metamorphosis to 2 months post metamorphosis. Even though a significant difference in days to metamorphosis did occur, a similar growth pattern was achieved after metamorphosis.

Table 7: Gonad Somatic Index. Tissue and body weight to indicate body condition at 2 months post metamorphosis. Ovarian somatic index = ovary weight / wet weight. Testis somatic index = testis weight / wet weight. Two standard deviations represented in parenthesis.

Treatment Level (mg/L)	Ovarian Somatic Index	Testis Somatic Index
0	0.641 (0.344)	0.136 (0.030)
10	1.132 (1.146)	0.121 (0.032)
50	0.629 (0.122)	0.11 (0.032)
100	0.612 (0.136)	0.108 (0.038)
200	0.566 (0.218)	0.167 (0.094)

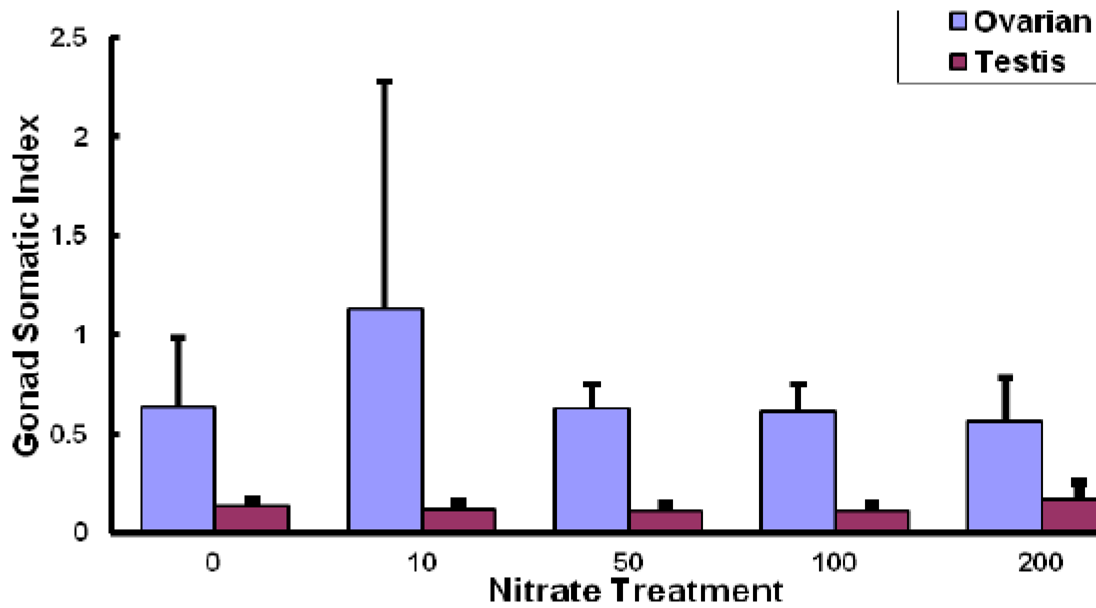

Figure 8: Gonad Somatic Index. Gonad tissue weight (mg) divided by the animal wet weight (g) and multiplied by 100, for each nitrate treatment (mg/L). Total tissue biomass does not describe the condition of the cells or tissue.

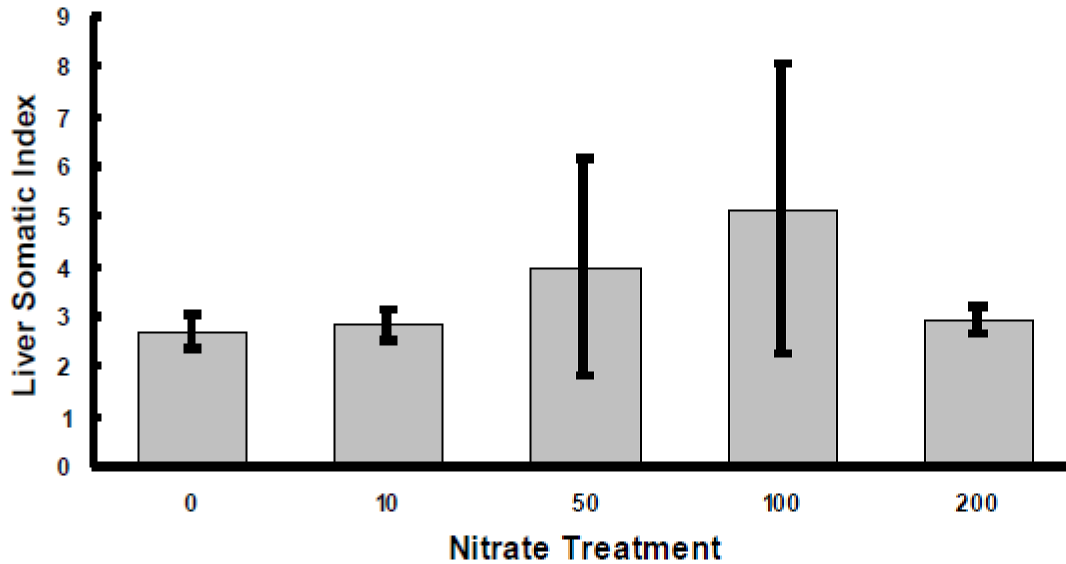

Figure 9: Liver Somatic Index. No significant difference between treatment groups. No trend was produced to indicate a dose response affecting tissue weight.

3.6 Kupffer Cell Density

The non-parametric ANOVA indicated a difference between the mean Kupffer cell densities among treatment groups. The GLM procedure confirmed the previous test ($X^2 = 72.56$, p = <0.0001, d.f. =8).

A chi-square test was performed on the sections defined as damaged (definition of damage in section 2.12 and 2.13) compared to non-damaged occurrences in the treatment groups when the slides were observed. A perceived difference in Kupffer cell density among treatments was observed (X2 = 22.0906, p = 0.0048, d.f. = 8). However, 50% of the observations had sample numbers less than 5 and thus questionable validity. The most damage occurred in treatment groups 10,10; 100, 100; and 200,0, therefore receiving no nitrate during post metamorphic development had less damage. However, groups 200,200 and 200, 0 do not fit the trend.

The mean number of Kupffer cells differed between the following treatment groups: 0,0 against treatment groups 100,100; 50,50; and 10,10. Treatment group 10, 0 differed from the following treatment groups: 50,0; 100,100; 50,50; and 10,10.

Table 8: Liver histology. Number of slides designated as damaged per treatment group.

Group	**Damaged**	**No Damage**
0,0	1	9
10,10	5	5
10,0	2	8
50,50	1	10
50,0	0	10
100,100	6	4
100,0	1	9
200,200	1	7
200,0	4	6

Table 9: Kupffer cell density between treatment groups to identify statistical variation. Ranked by mean values for each treatment group from lowest cell number to highest cell number. Separation Test, indication of statistical difference by letter. If two means do not share the same letter, they are significantly different, per Tukey's Studentized Range (HSD) test.

Treatment Identification	Mean Cell Value	Separation Test
10, 10	61.8	B
50, 50	62.9	B
100, 100	63.9	B
50, 0	67.3	AB
100, 0	73.4	A
200, 0	78.3	A
200, 200	78.8	A
0, 0	82	A
10, 0	85.5	A

Ranking the mean values and the information from the Tukey's separation test indicated which treatment groups were different. Treatment 10, 0 and 0, 0 had the highest number of Kupffer cells, and treatments 10, 10; 50, 50; 100,100 had the lowest number of cells.

3.7 Reproductive Gross Tissue Analysis

As tissue was excised to determine the GSI, gonads of particular histological interest were photographed and then examined for abnormal gonadal development. The gross morphology of the testes displayed in Figure 10 shows a non-normal elongated structure that was joined in the middle. Possible intersex animals were also observed (Figure 11). Pigmentation and a lobular structure was noted which is characteristic of a female ovary structure (Figure 11). Gross analysis of the reproductive tissues for the 10 animals assessed per treatment did not occur at a high enough frequency to produce enough robust data to determine a trend.

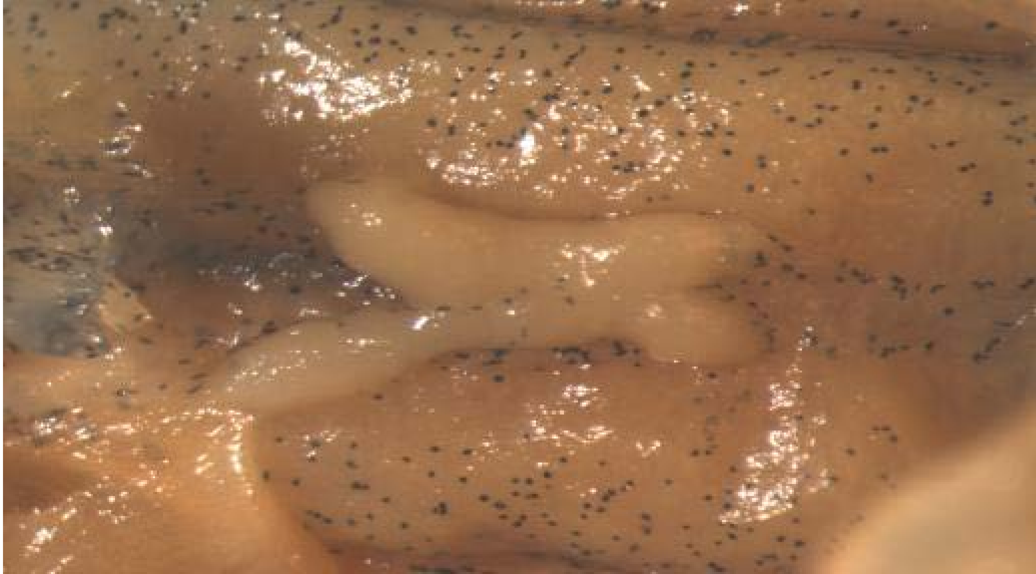

Figure 10: Gross Morphology Testis. ID: 10/10 #4, Identified as male. Testis removed with kidney. Testis are presented abnormally, the lobes of the testis are conjoined and elongated.

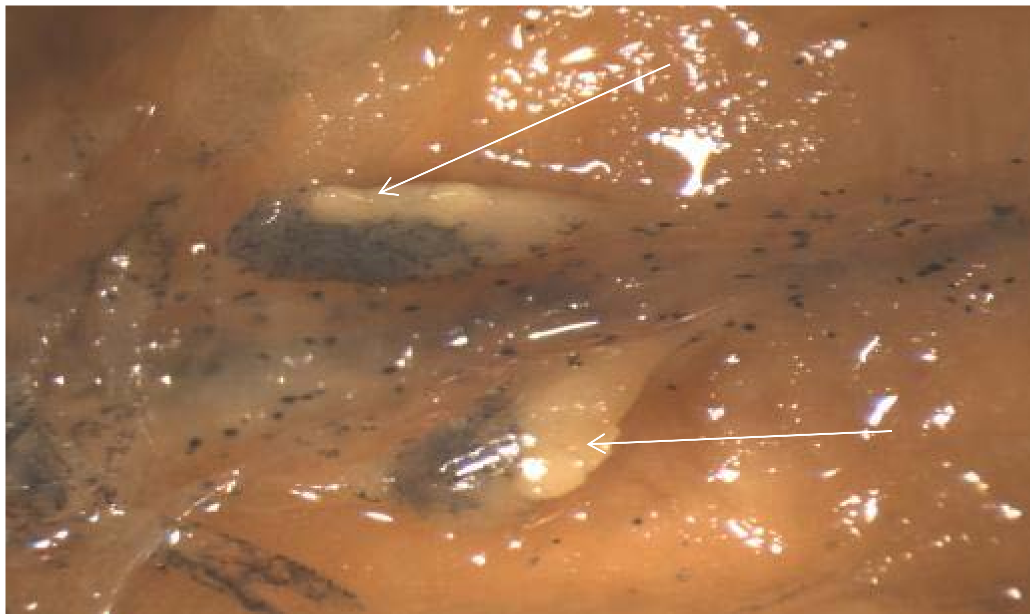

Figure 11: Gross Morphology Testis. ID: 10/10 #2, Identified as male. Testes are presented abnormally, lobes should appear solid in color (opaque), separated lobes attached ventrally to the kidneys via connective tissue. The testes presented appear with "fat bodies" (indicated by arrows), which is characteristic for female ovaries and discolored.

3.8 Fluctuating Asymmetry

No significant difference among treatment groups was indicated for fluctuating, directional, or anti-symmetry among the bilateral features analyzed. However, odd body

conditions were observed such as multi limb and loss of limb, lordosis. Figures 12 and 13 below show the most extreme of the deformities observed. The deformities were not determined to be associated with any specific nitrate concentrations but were considered to be sporadic throughout treatments. The Brown lab had not had a history of this type or frequency of deformities before.

Figure 12: Developed *Xenopus laevis* at two months postmetamorphosis demonstrate missing or extra limbs.

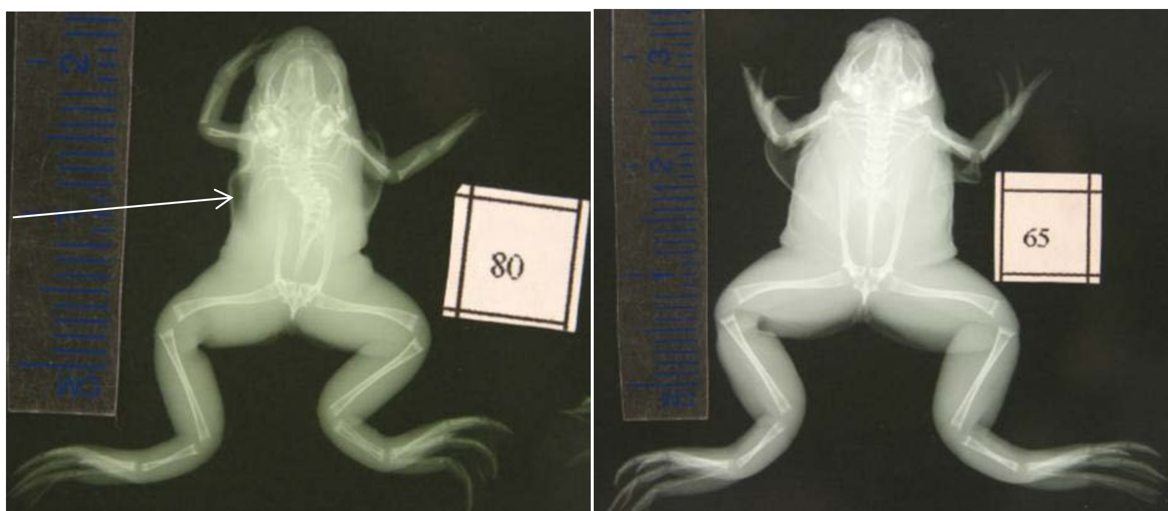

Figure 13: Lordosis, also known as sway back, indicated by arrow (Left) for animal exposed to 200, 200 mg/L, and non-lordosis (Right) for an animal exposed to 100,100 mg/L.

The outlier data from the FA measurements were analyzed for applicability and the removed during the first treatment of the statistical data. Articulation of the skeletal structure did prove difficult for structures that may have rotated such as the jaw and other head structures,

arms, and torso (reflected perhaps in the snout vent-length and in some animals the vertebral angles). As such, these measures were less robust and may be considered less reliable measures of other structures such as the tibia or fibia. The comparison of left to right symmetry, as mentioned above, found no difference of the between-side variation between treatments. The same number of significant figures for the sum of squares cannot be used because the sample sizes between the treatments varied significantly.

CHAPTER 4

DISCUSSION

The research design for this study was developed to increase duration of exposure to nitrate and eliminate confounding factors while providing an opportunity to collect behavioral data from the experimental animals. The protocol also provided an opportunity to collect histological data after the exposure period. Replicate measures were made to improve the statistical analysis and provide a robust population size. Evaluation of water quality provided confirmation that the test substance eliminated confounding chemical factors. Nitrate, nitrite, and nitrogen ammonia were all measured at expected levels within the test system but no significant accumulation of ammonia, a known aquatic toxicant, was found.

The purpose of the study was to determine the chronic effect of NO3- at environmentally relevant concentrations on the development of *Xenopus laevis* in an effort to model nitrate exposure in the environment. Results from this study add to a growing body of literature that focused on nitrate exposure effects on amphibians at larval, metamorphosis, and adult stages. This study, however, focused on nitrate exposure effects on amphibians from tadpole, metamorphic, to two months post metamorphic stages. This research focused on indicators of nitrogen stress such as body condition, hematocrit, and fluctuating asymmetry.

The results of this study do contrast with other relevant work regarding nitrate and amphibians and ads to discussion on species specificity and response to stressors. For example, Xu and Oldham (1996) recorded the effects of nitrate (100 mg/L) on *Bufo bufo* tadpoles for 24, 48, and 72 h and noted that the dose created a significant decrease in animal activity but not a clear reduction in food consumption or delay of development. They further noted that, at lower concentrations of 50 and 100 mg/L, metamorphosis started earlier and took less time to complete than in the control. Other key studies, such as Marco and Blaustein's (1999)'s work with *Rana cascadae*, provided that metamorphic rates shortened when exposed to 3.5 mg/L NO2-N. This is contrary to the data in section 3.4 showing that, for animals exposed to 200 mg/L of nitrate, their rate of metamorphosis was statistically significantly longer than that of the control. However, the

animals treated at 200 mg/L were not statistically significantly different from the other nitrate treatments.

The results were mixed regarding chronic effects, such as the longer time to metamorphosis at a high nitrate concentration (200 mg/L), and abnormal reproductive structures when excised during dissection at two months post metamorphosis. However, no clear dose relationship to any measured effect was observed. The sometimes prominent abnormalities such as missing or extra limbs and extreme lordosis cannot be directly attributed to developmental stability or the impact of a nitrate dose response. Such abnormalities may arise for reasons outside the scope of the research presented here, such as nematodes (Stopper, 2002) causing limb deformities, although it should be noted that there was no indication that the population used in this study were affected by nematodes.

Species chosen, use of nitrate excluding other impurities or compounds (such as ammonium), exposure technique, duration of the testing period and concentrations were developed to (1) encompass and facilitate other student research on the same animals (e.g. behavior) and (2) observe and evaluate if there were chronic exposure trends through what are thought to be sensitive periods of development for young frogs. Possible limitations in the experimental design should be addressed in a few areas: (1) exposure and concentration, (2) static versus flow-thru, and (3) measurement of body condition and fluctuating asymmetry. The concentrations used are likely to occur in the natural environment. A scenario that might be more beneficial would be one that has some variability between high and low exposure cycles and, therefore, might be more comparable to environmental scenarios such as seasonal fertilizer run-off from adjacent agriculture. Perhaps there are other guidelines for study design/experimental conditions that would be more tailored to this type of long exposure. An advantage to performing a chronic exposure study is the ability to observe delayed effects. Discontinued treatments not only provide a control for metamorphic to two months post metamorphic, but also a post-exposure observation period. Comparatively, acute tests conducted using the same species in other labs, have also been advantageous for capturing effects at specific life stages. For example, adult acute exposure for evaluating endocrine disruption (Guillete, 2005), an acute exposure may have been sufficient.

Utilizing a static renewal design versus flow-thru, for this particular study, may not have produced a different outcome. A flow-thru design would have simulated a consistent exposure of nitrate. The study design presented here simulates a pulsed exposure, although arguably the water

changes were frequent and show low temporal variability. The water quality remained consistently balanced, coupled with the water changing regime, enabled the frogs to be maintained for the duration of the study. Measurement for water cycles regarding the quality, were consistent with expected treatment levels and no build of ammonia was found in the study design.

Measurements of body condition can provide a reliable indicator of stress and relative success of a species. However, obtaining consistency with such measurements can be challenging. While these measurements are possible limitations to the study, the type of statistical analysis performed and the large sample size does minimize the possibility of human error. A multi generation approach may is more beneficial for evaluating body condition, because later generations may show developmental affects. The difficulty with a multi generation study is that it takes two years for the animal to reach sexual maturity.

From a histological perspective, there was no conclusive data that nitrate affected the animals' developmental homeostasis. However, anecdotally, animals did exhibit missing and extra limbs as well as perceived lordosis seemingly at a higher rate than previously experienced in the lab. The testis and ovary tissues were not fully matured so it may be beneficial during subsequent tests to evaluate these tissues in older animals.

Liver tissue evaluation did not demonstrate a clear dose response. However, a perceived trend was observed for those animals which were exposed to a higher concentration of nitrate the incidence of sections with unexplained inclusions or damage to the liver seemed to be higher. The shortfalls of the liver histology section should be considered for future studies and potentially have the slides re-evaluated after more in-depth training or understanding regarding the histopathological alterations observed. A different staining or slide preparation (peroxidase cytochemistry by light microscope in semithin plastic sections) method may have also produced different results by allowing a larger variety of cells to be surveyed and providing a more complete histological assessment. Other criteria that might be considered for later studies on liver pathology as an indicator of stress may include: occurrences of lesions, cirrhosis, hepatocellular necrosis, evidence of inflammation, and frequency of macrophages and their location (around periportal sites, midzonal or central area of the lobe). Another consideration may not be the distribution of Kupffer cells alone, but staining to look for indication of a functional deficit. The Kuppfer cell density results indicate a trend may exist between the increased number of Kuppfer cells in animals exposed to higher concentrations, but are

compromised by some apparent damage of the histology slide. Further research should be done to determine adequate sample size for this type of histology research. Also, more knowledge regarding the anatomical features of the liver should be gained in order to apply a more thorough survey of the tissue.

Literature shows conflicting outcomes when correlating environmental stress with FA and FA with fitness (Moller 1997, Pallmer and Strobeck 1988 and 1994, VanVallen 1962). FA was neither indicated nor eliminated as a useful biomarker in this study. Alternate measuring techniques may prove more effective at detecting smaller variations. Perhaps more sensitive software would also prove more advantageous. The objective was to decrease measurement error in the small characters by using a radiograph which was able to provide a defined bone end for measurement. The radiograph method developed was beneficial from the perspective of overall articulation of the skeletal structures and documentation of the animals, but the age of the animal (bone calcification) may have been a limiting factor. X-rays allowed us to evaluate gross pathology but the detail was not fine enough for minute deviations. In previous work with *Xenopus* (Gurung, 2004), a tissue clearing and bone staining technique was used to measure bone asymmetry. If the bones were disarticulated in that study, deformities such as lordosis in the skeletal structure would have been less clearly visible.

During the FA measures, bones were measured twice for each animal. The statistical power of the analysis was sufficient because the sample size was considered large for this type of study and analysis (Palmer and Strobeck, 1992). A summary of growth and development indicated no statistical gross differences between nitrate treatment levels. That effects were not seen in this study may be attributed to the species used. The species may not be sensitive to the particularly low concentrations used over a chronic period and/or the stage of development was not receptive or able to express/reflect the stress of chronic nitrate exposure. It is possible that the indicators monitored are extremely canalized and therefore do not express a response within this generation, but the species would possibly show an effect in subsequent generations. A variety of studies indicate that bones are a poor indicator of stress and that other tissues may more readily display signs of stress at the time of exposure onset. Differences between long and short bone landmarks (Bailey and Byrnes, 1990) do affect the accuracy of measurements. To improve measurement accuracy, the sampling method included long and short bones from the entire skeleton. Appropriate metrics for effective sample size regarding FA have been addressed

in literature by VanValen (1962) and Palmer (1996) and the protocols and statistical analyses they outline as a means to minimize ME were applied within the research presented here.

As indicated by other researchers (Allenbach et al. 1999, Allenbach 2011), consistency in the field and laboratory for FA determination methods are needed. The fluctuating asymmetry aspect of this study was unique regarding the method of measurement and can be debated as an effective indicator of stress. The naturally-occurring symmetry or small variation that occurs in an organism independent of stressful development may reduce the probability of observing significant FA results (Allenbach, 2011). Correction factors were applied based on the control animals and pixilation of the image during measurement.

Any left-right asymmetry observed at two months post metamorphosis may have originated during the larval stage due to developmental noise or other unexplained geometrical variance (Levin, 2004). The calcification of the bones at two months post metamorphosis was determined to be still premature compared to adult long and short bones and may have been a limiting factor.

Questions that remain for the effects of nitrogen contamination on amphibian fitness and population growth include: (1) refining the methods or statistical tests for determining FA following nitrate exposure, (2) of how nitrate affects liver function and histology and why some of the indicator symptoms found in other higher species were not apparent in the amphibians tested in this study, (3) which test (acute or chronic) is more advantageous when assessing body condition (including endocrine disruptions) for nitrate effects

The purpose of this study was not to draw a corollary line from lower to higher species regarding effects. However, what is interesting is the lack of expected response, in particular the hematocrit of the *Xenopus*, which was contradicted by literature for humans exposed to nitrate. It would have been expected that some of the known effects (in other species) would have been mimicked and observed in the tested amphibians at some level. This leads to the concept that *Xenopus* may not be the correct corollary/sentinel species for other areas of research regarding nitrate (for example endocrine disruption) unless the mechanisms of action (toxicokinetics) are the same. This needs to be addressed by further research. Before making strict suppositions that lower and higher species are analogous for specific endpoints, modeling may be advantageous.

The evolution of the field of toxicology now includes a heavy discussion of endocrine disruption and the theories presented by Hayes and Gillette increase the evidence and the need

for continued evaluation of nitrate at chronically low levels in the environment. As the theory and understanding of the effect of the environment play on endocrine disruptors evolves, the testing and detection methods in situ will undoubtedly increase in accuracy and efficacy. The data and its current treatment in this study did not evaluate nitrate as an endocrine disruptor. The evidence of endocrine disruption as an effect of chronic nitrate exposure may be indicated though with a detailed histological evaluation of the reproductive tissue that was excised and preserved onto slides. If the frogs were allowed to progress into reproductive maturity, perhaps their breeding success in future generations would also have served as an indicator of nitrate as an endocrine disruptor in the species.

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APPENDIX A

NITRATE CONVERSION FOR NITROGEN AND DOSING

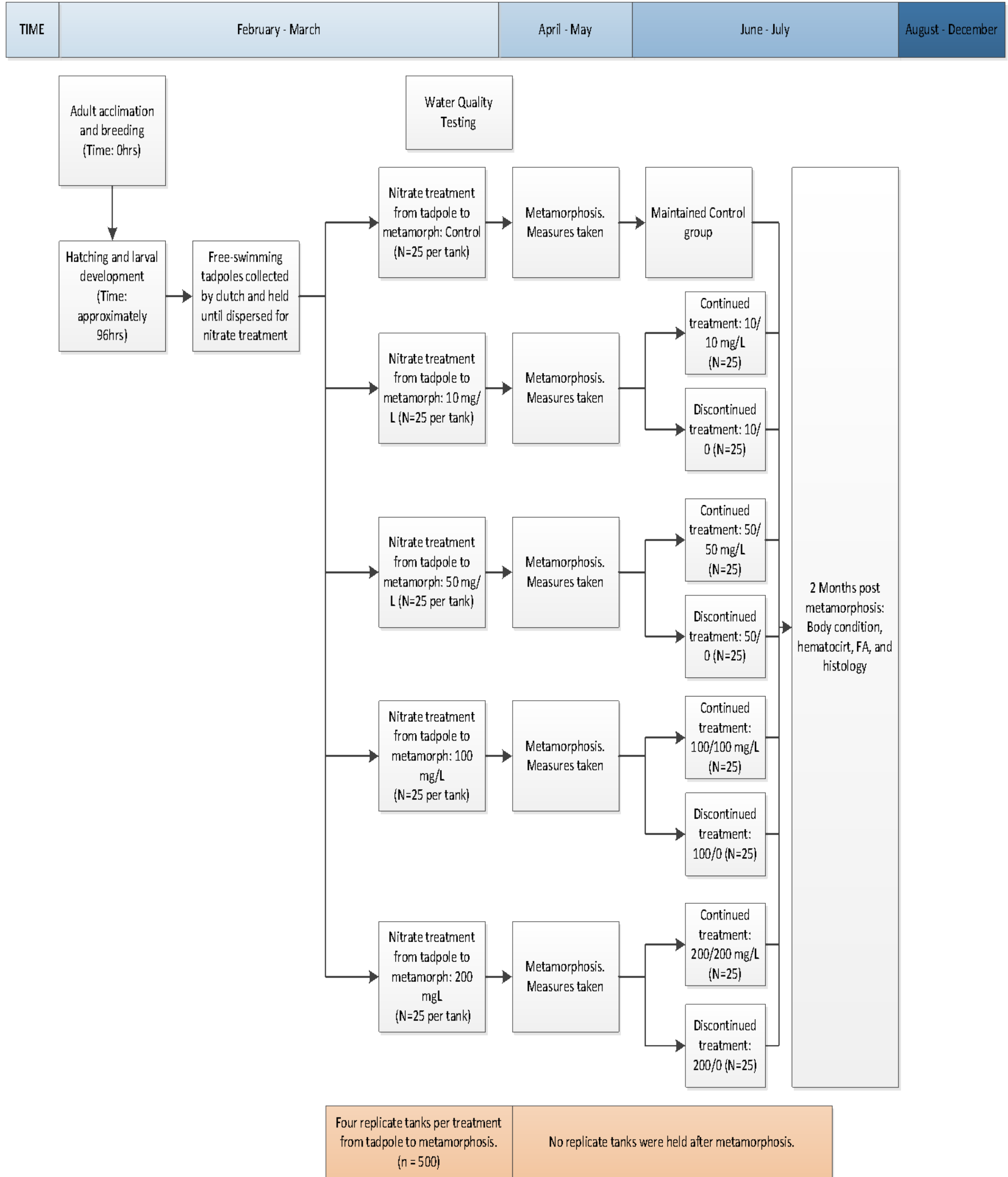
1 gram $NaNO_3$ equivalent to 73% NO_3 or 1.37 g of $NaNO_3$ for every 1 g of NO_3.

1 ppm of NO_3 is 0.00137 $NaNO_3$ to 1 Liter of water.

NO3 Exposure	NaNO3 in Dilution (mg/L)	Dose for 4 Liters of Water (g/L)	Dose for 5 Liters of Water (g/L)
0	0	0	0
10	13.7	0.548	0.685
50	68.5	0.274	0.342
100	137	0.548	0.685
200	274	1.096	1.35

APPENDIX B

STUDY DESIGN



APPENDIX C

LIST OF MEASURES AND DIAGRAM OF X-RAY FOR FLUCTUATING ASYMMETRY MEASURES

Identifying Number (Left, Right)	**Abbreviation**	**Feature**
1, 18	TWU	Tibia width upper length
2, 17	TFWB	Tibiofibula width bottom
3, 16	TFO	Tibiofibula outer length
4, 15	TFI	Tibiofibula inner length
5, 14	TFWT	Tibiofibula width top
6, 13	FEBW	Femur bottom width
7, 11	FEI	Femur inner length
8, 12	FEO	Femur outer length
9, 10	FET	Femur top width
27, 28	HL	Humerus length
30	SV	Snout-vent length
a, b, c	VA	Vertebra angle
25, 26	JAW	Jaw
29	S	Spine length

2
1
CM
25
26
24
21
22
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a
30
29
27
28
b
c
9
10
12
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11
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17
18
3
4
2
1
144